The Physiological Significance of the Water Soluble Components of Nervous Tissue.

by

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A thesis submitted for the degree of Ph.D. in the University of Edinburgh.

Preface.

The experimental work reported has been carried out in the Physiology Department during the last three years under the supervision of Miss C.O.Hebb and Dr. P.Eggleton.

With the exception of the preparation of coenzyme A and acetate-free ATP the experimental methods used have been reported previously.

No previous reports have been published on the efficacy of acetate in acetylcholine synthesis. The utilisation of acetate by this system has been inferred by workers engaged in this field. The suggestion of Lipton and Barron (1946) that citrate dismuted to acetate which is then utilised for choline acetylation has been found to be correct.

Comline's spleen enzyme has been a useful tool for working out the structure of the coenzyme since it breaks the coenzyme into fragments which can be resynthesised by a liver enzyme.

The structure of the coenzyme is discussed in the light of reports in the literature and the experimental evidence reported here.

Unless acknowledgement is made in the text, all work in this thesis has been carried out by me.

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Introduction.

In vitro studies demonstrate that as little and probably much less than 1mg. of protein derived from the water soluble fraction of nervous tissue can, under well defined conditions, catalyse the synthesis of up to 100μg of acetylcholine per hour. This fact, among others, has led various workers to postulate far-reaching hypotheses for the function of acetylcholine in nervous tissue.

Acetylcholine and Axonal Conduction.

Calabro in 1933 first suggested that acetylcholine may play an important part in the processes of excitability and conduction in axons. More recently, Nachmansohn proposed that "the potential difference observed during nerve activity may be closely connected with the metabolism of acetylcholine at or near the surface of the nerve cell, the metalolism being only quantitatively more important at synapses". Fulton and Nachmansohn (1943) have generalised the theory by stating that the release of acetylcholine is the elementary process underlying the propagation of the impulse along the peripheral fibres.

In support of the theory in question the following arguments have been developed by Nachmansohn:

a) Choline esterase is localised at the neuronal surface:

b) the nerve action potential is connected with alterations of the nerve membrane:

c) the electric organs of fishes have an extraordinarily high concentration of choline esterase:

d) there is a close correlation in these organs between the voltage of discharge and the concentration of choline esterase.

From such data Nachmansohn has inferred that the alterations of the nerve membrane that take place during activity, are intrinsically connected with the release of acetylcholine.

Further evidence for this theory has been sought by the study of the action of anticholine esterases on nerve conduction. Neither prostigmine nor eserine (Cowan (1938), Cantoni and Loewi (1944)) affects conduction.

Diisopropyl fluorophosphate, a powerful anticholine esterase is reported to have contradictory actions on nerve conduction. Crescitelli, Koelle and Gilman (1946) found that nerves treated with this substance could conduct impulses normally although their choline esterase activity was zero. Bullock et al. (1945) contradicts this report stating that there is a parallelism between the degree of reversibility of action potential abolition and the choline esterase activity. Boyarsky, Tobias and Gerard ((1947) then demonstrated however, that in frog nerve

exposure to DFP for a time long enough, and in concentration strong enough to inactivate the choline esterase had no effect on the action potentials. When the nerve was exposed to unnecessarily high concentrations of DFP the action potentials at first decline and then finally disappear.

Barnes and Beutner (1946) in model experiments have shown that acetylcholine produces a marked boundary potential at the interphase between a saline solution and oil and have suggested that a similar effect is the basis of the development of the spike potential of nerve.

Instead of arguing from the standpoint of choline esterase activity some workers have made direct experiments with acetylcholine. In all cases (Bronk 1938; Lorente de No 1944) the results have been negative.

To these negative results Rothenberg, Sprinson and Nachmansohn (1948) attributed the lack of penetration of acetylcholine into the interior of nerve fibres. However, Gerard, Libet and Cavenagh (1949) have reported that acetylcholine does penetrate into resting giant squid axons.

However the active membrane probably allows the penetration of acetylcholine since it is well known that there is an outflow of the mediator at cholinergic synapses. The inability of acetylcholine to

influence the properties of axons is therefore an important objection to Nachmansohn's theory. In fact until it is shown that the choline esterase of nervous tissue is more specific for acetylcholine than for other enzyme systems, the study of the action of anti-choline esterases on action potential propagation is not relevant.

Acetylcholine and Synaptic Transmission-

That a chemical agent might be the transmitter at ganglionic synapses was first suggested by Kibjakov (1933). Feldberg and Gaddum (1934) showed that the agent released in Kibjakov's experiments was acetylcholine. Direct evidence that motor nerves of striated muscles are cholinergic was obtained by Dale and Feldberg (1934).

In the elaboration of this theory the following premisses had to be adopted.

1) Acetylcholine is released by presynaptic nerve endings.
2) Acetylcholine can elicit synaptic and endplate potentials. These potentials can in turn initiate post synaptic impulses.
3) The amounts of acetylcholine released by the nerve impulses are sufficient to elicit synaptic and endplate potentials of an amplitude that is above the threshold of the post-synaptic elements.

4) High concentrations of acetylcholine depress post-synaptic elements instead of stimulating them.

5) The output of acetylcholine per nerve impulse decreases progressively if the stimuli are applied with a frequency higher than a certain relatively low rate.

6) Prolonged stimulation of a presynaptic nerve fibre at a moderate rate (60 per sec.) leads first to a decrease of the output of acetylcholine per impulse but later to an increase of this output.

7) Choline esterase destroys acetylcholine so rapidly that the synaptic and endplate potentials elicited by the mediator are below the threshold of the post-synaptic elements at the end of the refractory period of these elements.

All these premisses are supported by experimental observations. Further all the instances inexplicable on the bases of the electrical theory can be explained. The chemical theory does not include the obscure role of K^+ ions in the process of transmission. The post-tetanic effects are undoubtedly not due to acetylcholine, since they can endure long after the mediator liberated by tetanic stimulation has been destroyed. It seems quite probable (Wilson and Wright 1936) that the mobilisation of K^+ ions may account for post-tetanic enhancement.

In short, the role of acetylcholine as mediator

in synaptic transmission is well established; its action in axonal conduction is not understood.

A means of approach to the physiological role of acetylcholine in axons would be given by data showing whether acetylcholine can be synthesised and in what quantities.

Many reports have appeared in the literature on the capability of nervous tissue to synthesise acetylcholine. In view of the many contradictory reports by various workers and because a mechanism has not been proposed with any conviction, a resolution of this problem forms part of the work of this thesis. When the conditions required for the demonstration of acetylcholine synthesis are well defined and when they are shown to be founded on logical premisses then it will be possible to investigate the spatial distribution in the nervous system of the enzyme, choline acetylase.

Unless the mechanism of synthesis is well defined it is probable that surveys of the occurence of choline acetylase in the nervous system are not correct, especially when ad hoc methods for the demonstration of acetylcholine synthesis are used.

Acetylcholine Synthesis.

As early as 1924 Aberhalden and Paffrath attempted to reverse the usual hydrolytic action of the

enzyme choline esterase but they had little success even with choline and acetate concentrations far above those found physiologically. In 1937 Brown and Feldberg added substances to the fluid perfusing a superior sympathetic ganglion and measured the output of acetylcholine in the perfusion fluid or the acetylcholine content of the tissue. Choline increased the rate of synthesis in some experiments. In a similar manner Kahlson and McIntosh (1939) noted the accelerating effect of glucose and the inability of acetoacetate and acetate to influence this synthesis.

About the same time two groups of investigators, (Quastel et al.,(1936) and Stedman and Stedman (1937), evolved methods which, in contrast to those of Feldberg and Brown amd Kahlson and McIntosh, allowed some examination of the mechanism involved in the synthesis of acetylcholine.

Quastel et al. studied the synthesis of acetylcholine by brain slices or pulp in saline media while the Stedmans evolved a technique in which acetylcholine synthesis was examined in brain pulp after grinding the material with either chloroform or ether. In both cases eserine was added to inhibit the breakdown of acetylcholine by choline esterase. In general the principal difference between the results from these two methods was found in the effect on the

synthesis of the addition of various substances to the media.

Quastel et al. showed that the synthesis of acetylcholine was closely associated with respiration and did not occur under anaerobic conditions. They showed further that under anaerobic conditions the formation of acetylcholine was accelerated by the addition of glucose and to a smaller degree by the addition of either lactate, pyruvate, and glycerophosphate or glutamate. On the other hand, acetate, succinate, acetoacetate, hexose diphosphate and ketoglutarate did not influence acetylcholine synthesis. Moreover, the addition of choline increased the amount of acetylcholine formed by brain slices.

Stedman and Stedman while agreeing that acetate had no effect in their system found that pyruvate had a considerable inhibitory effect on the synthesis; on the other hand, with this method acetoacetate increased the yield approximately 50%.

Many points of difference between the results of Quastel et al. and Stedman and Stedman were clarified by Feldberg (1945) who showed that while ether does accelerate the synthesis of acetylcholine by vacuum dried brain powder, at the same time it has an inactivating effect on the enzyme system responsible for synthesis. Since an increase in temperature increases the destruction of the enzyme systems to a greater

extent than it accelerates the acetylcholine synthesis the optimal conditions for the demonstration of the activating effect of ether are to be found at room temperature and not at 37°.

The position is too confused for one to form any definite opinion or to adjudicate between the arguments of Quastel on the one hand and Stedman on the other. In any case, the experimental data given by tissue slice techniques made further advances possible.

Nachmansohn and Machado (1943) studied the effect of ATP on acetylcholine synthesis in homogenised brain tissue. They found, under anaerobic conditions, that ATP stimulated the synthesis of large amounts of acetylcholine. These authors named the enzyme responsible for acetylcholine synthesis "choline acetylase" and they also presented evidence that this enzyme belonged to the SH-sensitive froup. In 1945 Nachmansohn and John found that on dialysis homogenised brain extracts lost their activity but that partial reactivation occurred on addition of either glutamate or citrate. A strong inhibiting effect was found when pyruvate or other keto acids were added to the incubation medium.

A great advance came when Feldberg and Mann (1945-6) showed that acetone dried brain tissue provided not only a much more active enzyme preparation yielding more constant results but also the powder

was a form in which the enzyme could be stored. They showed that acetone drying destroyed most of the choline esterase activity, that K^+ accelerated the synthesis of acetylcholine by these extracts and also that the synthesis could be studied under aerobic conditions provided that cysteine or glutathione was present. The effect of addition either alone, or in combination of citrate, ATP and boiled brain extracts to the incubation mixture was studied with both dialysed and undialysed enzyme preparations. The citrate effect with either enzyme preparation was most marked. ATP and boiled brain extract exerted their most marked potentiation with a dialysed enzyme preparation. In the same paper Feldberg and Mann deduced the presence of a cofactor (activator), other that ATP or citrate

Nachmansohn and Berman (1946) later studied this factor and gave a method for its preparation. Even with such systems catalysing much acetylcholine formation both groups of workers agreed that the addition of acetate had no effect of the synthesis.

About this time Lipton and Barron (1946) confirmed the results of Feldberg and Mann but contributed nothing new but the suggestion that citrate was acting as a source of acetyl radicals.

Feldberg and Hebb (1947) then demonstrated that the potentiating effect of citrate on acetylcholine formation was, in both dialysed and undialysed extracts dependent on the presence of Mg^{++} or Mn^{++}. They fur-

ther differentiated between synthesis of acetylcholine in the presence and absence of citrate. Creatine phosphate could replace ATP provided adenosine monophosphate was also added.

During the years 1945-7 Lipmann had been examining the mechanism of acetylation of sulphanilamide in extracts of pigeon liver. He showed that this acetylation too required the presence of ATP (Lipmann 1945) and thermostable factors present in boiled tissue extracts. One of these has a nuclectide nature and he termed it the "coenzyme of acetylation". For optimal activity this "coenzyme of acetylation" required the presence of citrate, cysteine and ATP. It seemed probable that this coenzyme of acetylation was identical with the activator of Feldberg and Mann (1945-6). The sulphanilamide system did differ however from that involved in the acetylation of choline in that acetate had a powerful accelerating effect on the former but, as mentioned above, it had no action on the latter. Two suggestions have been advanced to account for the difference between the two systems. Nachmansohn (1948) has suggested that the lack of the effect of added acetate was due to its presence as a contaminant in the ATP. Kaplan and Lipmann (1948) think that citrate exerts its effects by binding Ca^{++} which is a necessary factor for ATPase activity. Lipton and Barron (1946) on the other hand have suggested that citrate can yield "active" acetate and

and oxaloacetate, thereby accelerating synthesis by providing a substance for the acetylation of choline.

It is convenient at this point to correlate some of the more modern data with the earlier observations of Quastel et al. and those of Stedman and Stedman. It is probably correct to assume that the supply of respiratory energy shown by Quastel et al. (1936) to be necessary for Ach synthesis was replaced in the later experiments by the phosphate bond energy of ATP. Thus the accelerating effect of glucose in Quastel's experiments was probably associated with stimulation of glycolysis and oxidation in the brain cells to provide a supply of ATP.

The system used by Stedman and Stedman (1937-9) bears a close resemblance in its various reactions to those found with homogenised brain or saline extracts of acetone dried brain. This is illustrated by the inhibiting effect of pyruvate in both systems (Stedman and Stedman 1939, Feldberg and Mann 1945-6, Nachmansohn and John 1945) and the small respiratory activity found in both cases (Feldberg and Mann 1945-6, Jowett and Quastel 1937).

This historical review of the work on Ach synthesis demonstrates clearly that with the development of experimental technique the system required for optimal conditions of synthesis, although

complex, became relatively well defined in content. Using dialysed enzyme preparations, synthesis of Ach does not occur (with choline, K^{+}, cysteine, Mg^{++}, eserine) unless one or other, or a combination of two, or all three of the following substances is added. These cardinal substances are 1)Activator; 2)Citrate; 3)ATP.

The purpose of the work contained in this thesis has been, in the first place, to identify absolutely the substance responsible for the action of the activator fraction and secondly, to demonstrate the function of these three substances.

EXPERIMENTAL METHODS.

Synthesis of Acetylcholine was studied in saline extracts prepared from acetone-dried brain of guinea pigs and rabbits, the gut of rabbits, the gut of rabbits and a dog. It was also studied in ammonium sulphate fractions from the rabbit and guinea pig brain.

Preparation of acetone-dried tissue.

Brain:- The guinea pigs and rabbits were killed by a blow on the neck and the brain removed by transverse section immediately in front of the cerebellum. The cooled brains were ground in a mortar with acetone at 0° or -10°C. The resulting powder was collected on a Buchner funnel and dried in air. The powder was stored at 2°C in a vacuum desiccator over CaCl2.

Duodenum:- The duodenum of rabbit and dog was removed and stretched by insertion of a test tube into the lumen. The outer connective tissue was removed and the circular and longitudinal muscles removed and ground in ice cold acetone.

Pigeon liver:- The livers of twelve pigeons were removed after decapitation and bleeding and cooled to -5°C. The frozen tissue was minced in a Latapie

mincer. Twenty volumes of acetone at -5° C was added to the mince and the mixture ground in a large pestle and mortar for two minutes. The insoluble matter was collected on a sixteen centimetre Buchner funnel and it was washed with more acetone and ether at -5°C. After drying for thirty minutes in air the powder was removed and stored in a vacuum desiccator over $CaCl_2$ at 2°C.

Horse and rabbit spleen:- The organ was removed and the pulp dissected away from the connective tissue trabeculae. The pulp was cut finely with scissors and ground in ice cold acetone.

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Preparation of ATP.

The nucleotide was prepared according to Kerr's modification (1941) of Lohmann's method (1928) and also according to Potter and Le Page (1949) where it was noticed that magnesium anaesthesia did not improve the yield. The samples were analysed for inorganic, seven minute hydrolysable and total phosphorus by the method of Fiske and Subbarow (1925). The preparations used were never less than 97% pure.

Acetate-free ATP was prepared by two methods:
1) by solution of the crude barium salt in N HCl and reprecipitating twice with barium nitrate.
2) by the substitution of barium nitrate for barium

acetate in Kerr's procedure. The lower solubility of the nitrate necessitates the use of three times the volume of 8% barium nitrate for precipitation. ATP prepared by this method never assayed less than 99%.

Before use, the barium salt of ATP was dissolved in 0.1N HCl and the barium removed with an excess of sodium sulphate. After centrifuging off the barium sulphate it was washed with 0.1N HCl and the combined supernatants were neutralised.

The Activator or Coenzyme of Acetylation.

The substance was prepared by boiling saline extracts of acetone dried guinea pig brain.

Two methods have been developed for purification of the activator. One of these methods allowed us to establish the identity of the activator of Feldberg and Mann (1945b) with the coenzyme of acetylation.

Preparation of CoA from Yeast.

(A). PHENOL EXTRACTION OF THE YEAST.

(1). 10 lbs. of Bakers' Yeast is extracted with 9 litres of Tap water. The yeast is gently crumbled on a large sheet of paper before adding to the water in a large pan. The water is heated

until boiling before any yeast is added; this is done at such a rate that the temperature does not fall lower than 85°- 90°C. After the addition of all the yeast the mixture is kept at as near 85 C as possible for 10minutes (temperature limits 85°- 90°C.) Note: If pan overflows transfer excess to flask and treat separately until centrifuging.

2).
2). Cool insink with water plus ice.

3). Spin off in serum centrifuge; discard precipitate but preserve the supernatant, i.e. the Kochsaft (approximate volume 8.4 litres).

Note: The preparation may be left overnight at this stage.

4). Add (NH4)SO4 to the supernatant after pouring off from the precipitate. The final concentration should be 500gms. (NH4)2SO4 per litre of Koch- The (NH4)2SO4 should dissolve quite easily.

5). Preparation of the Phenol.
Add approximately 5% water to the Phenol (detached crystals), warm until the Phenol liquifies. 1lb. of Phenol should yield about 500ccs. of liquid.

6). Add Phenol to the Kochsaft. 1 litre Phenol to 8

8 litres of Kochsaft. i.e; 1.125 litres of Phenol to 10 litres of supernatant. The mixture should be gently stirred while adding the Phenol- but NOT too vigorously or the Phenol will never separate out again.

7). Pour the mixture into large conical flasks, and allow to stand until the Phenol has separated off as a fairly definite layer. This should take about 2 to 3 hours; (for the above quantities two 6-litre flasks are convenient). Leave to extract for three days.

8). Suck off the Phenol layer as thoroughly as possible and centrifuge the resultant mixture. After about 15 minutes spinning it should be possible to suck off the clear Phenol layer; the colour should be brownish-yellow from the first extract to yellow from the third.

9). Add the same quantity of Phenol as before and repeat extraction.

10). Again re-extract with fresh Phenol. The separation on this occasion must be thorough and if the Phenol layer does not come to the narrow part of the flask it is advisable to separate from a complete flask and use the separated material to fill up the flask, allow to stand and then

separate the last portion with greater efficiency.

NOTE. The preparation may be left for one week at any stage in the Phenol extraction; in fact it is an advantage to do so as it gives a better separation of the Phenol and watery components.

11). Add to the separated Phenol an equal volume of ether and shake up in a separating funnel with 10 lots of 60ccs. of distilled water, (i.e. for the 10lbs of yeast used). The watery layer is then separated off giving a dark brown solution of the dinucleotides.

12). The pH of this solution is adjusted until it is just acid to Congo Red, i.e. it gives a blueish-brown colour. 2N HNO_3 is used to adjust the pH - about 20ccs. are required.

13). Add 9ccs. of 30% $AgNO_3$ to the acidified extract.

NOTE. After spinning off the precipitate of the silver salt of the flavine-dinucleotides the addition of more $AgNO_3$ produces a further precipitate in the immediate vicinity of the drop which dissolves on shaking. On standing overnight a yellowish coloured precipitate separates out. This is probably NOT a flavine dinucleotide. and can be spun off, the supernatant collected and the precipitate is not worked up.

14). Allow to stand for about 30 minutes.

15). Spin off the silver precipitate.

NOTE. There appears to be more precipitate than there ought to have been at this stage, probably due to contaminating polynucleotides.

16). Wash the precipitate with approximately 20ccs. of distilled water to which a drop of 2N HNO_3 has been added to bring to the same pH as before.

NOTE. Preparation can be left overnight at this stage.

17). Spin off the precipitate:add the supernatant to that from (15).

18). Suspend the silver precipitate in 50-100ccs. of distilled water (the exact volumes do not really matter), but by washing bring into one large (200cc) centrifuge tube.

19). Add strong 20% KCl until the precipitate more or less comes into solution.

20). Spin off the undissolved dark material.

21). Wash the undissolved precipitate with a small quantity of distilled water containing a little KCl and combine supernatant fluid. The precipitate can be discarded.

22). Sufficient 2N HNO_3 is added to the supernatant to make it acid - about pH 3 or 4 - the AgCl is

precipitated by this procedure.

23). Spin off the precipitate and keep the supernatant.

24). Wash the AgCl precipitate with water acidified with 2N HNO_3.

25). Combine the supernatants. The dinucleotide solution should have a volume of approximately 80ccs. Neutralise the solution.

26). Preparation of the p-Cresol is almost the same as that of Phenol. Warm with water, transfer to a separating funnel, wash with a solution of $(NH_4)_2SO_4$ containing 50gms. per 1000ccs. Leave the Cresol in the separating funnel; it may have a reddish tinge.

27). Add 40gms. $(NH_4)_2SO_4$ to the 80ccs. supernatant. (i.e. 50gms./1000ccs.). Shake up to dissolve and spin off any precipitate which may appear.

28). Take the supernatant and add to the 5ccs. of washed Cresol in the funnel and shake up.

29). Allow to separate and take off the dark brown Cresol solution. First remove the aqueous solution and then pour the Cresol solution out of the top of the separating funnel.

30). Repeat extraction with 1cc. of Cresol and add to the 5ccs. of Cresol already used.

31). Wash the Cresol with acid $(NH_4)_2SO_4$ three or four times.

Acid ammonium sulphate : 50gms. salt, 80ccs. H_2O, and 20ccs. N H_2SO_4.

Discard the wash solution each time.

32). Add an equal volume of ether to the Cresol (i.e. 6ccs.) and shake with 2cc. lots of water. This produces the flavine adenine dinuclectide solution The darkest extracts of approximately 9ccs. should be poured into a small centrifuge tube. Neutralise the solution.

33). Allow to stand and if there is any precipitate spin off. (Dark brown precipitate).

Wash any precipitate twice with a very small quantity of water and add to the solution.

34). Extract with water saturated S-Collidine until the upper layer no longer fluoresces green under ultra-violet light.

Preparation of s. Collidine. This solvent was purified according to Partridge (1948). After saturation with water it was used to separate flavine adenine dinucleotide from the activator.

Acetyl phosphate was prepared as the lithium salt by the method of Lipmann and Tuttle (1948).

Acetyl metaphosphate was prepared by a modification of the above method when silver metaphosphate was treated with acetyl chloride in ether in the presence of metaphosphoric acid.

L Cysteine was obtained from Light Bros. It was recrystallized after removal of inorganic phosphate with baryta.

The anion-exchange resin De Acidite E was supplied by the Permutit Water Coy. Before use it was soaked in water overnight. After backwashing and regeneration with N NaOH it was charged with chloride ions from a N HCl solution and washed acid free with distilled water.

Preparation of Enzyme Extracts.

Choline acetylase. Crude extracts of acetone dried tissue were ground with 0.9% NaCl to give a concentration of 50mg of powder per ml. The mixture was centrifuged and the supernatant used. In some experiments the supernatant was treated with about one third of its volume of resin. After half an

hour the resin was removed by filtration through glass wool. In other experiments the supernatant was dialysed against at least two litres of 0.9% NaCl for up to 60 hours at 2° C. The saline was changed at least twice during this period. Fractionated extracts were made with ammonium sulphate essentially according to Nachmansohn et al (1949).

Pigeon liver enzyme. The crude extract was prepared and aged according to Kaplan and Lipmann (1948).

Fractionated extracts were prepared with ammonium sulphate. The method adopted was as follows: the crude aged extract was brought to 35% ammonium sulphate saturation at 0° C. The resulting precipitate was removed by spinning or by filtration with a Whatman No 15 filter paper after the suspension had stood at 0° C for one hour. The supernatant or filtrate was then brought to 80% ammonium sulphate saturation at 0° C. After standing for one hour the resulting precipitate was collected by filtration and dissolved in half the original volume of 0.02 M $NaHCO_3$. Sometimes this process was repeated. The enzyme solution was dialysed overnight at 2° C against two litres .02 M $NaHCO_3$ and 1 millimol cysteine.

A preparation of the sulphanilamide condensing enzyme was prepared essentially according to Lipmann

(1950). Since he does not give the details of preparation we report the method developed here. The crude aged extract (10% acetone powder) was diluted with an equal volume of 0.02 M NaHCO3: thus the ionic strength was 0.04 and acetone at -20° C was added to bring the concentration of acetone to 40% by weight. After standing for 1 hour at -5°C the resultant precipitate was removed by filtration. The filtrate was brought to a concentration of 60% acetone and the precipitate collected and dissolved in half the original volume of 0.02 M NaHCO3. Acetone was removed by dialysis against six litres 0.02 M NaHCO3 and 1 millimol cysteine.

Spleen enzyme. The acetone dried tissue was ground in sufficient saline to bring the concentration to 100mg. per ml. The insoluble material was removed by centrifugation. The supernatant was dialysed against several changes of 0.9% saline at 2° C.

Intestinal phosphatase. The procedure adopted was essentially that described by Hawk (1947).

Conditions of Incubation.

1) Acetylcholine synthesis with crude enzyme.

The incubation samples contained, unless otherwise stated, the following:- 3mg. choline, 6mg. KCl, 4.5mg. cysteine, 0.5mg. eserine sulphate, 2-4mg. MgCl2 and 0.4mg ATP phosphate calculated as pyrophos-

phate P(P7). Where necessary 0.3- 0.5ml. M/15 sodium phosphate buffer (pH7) were added. The samples had a final volume of 4.5cc., being adjusted with 0.9% NaCl to the required volume.

In all cases, unless otherwise stated, the samples were incubated aerobically for 1 hour at 37° C.

2) Fractionated brain enzyme was prepared according to Nachmansohn et al. (1949).

3) The acetylation of sulphanilamide and estimation of coenzyme A was conducted according to Kaplan and Lipmann (1948). The final volume was usually adjusted to 1.1ml. When fractionated extracts were used, citrate, as a rule, was omitted. Otherwise the conditions were as above.

4) Brain-liver coupling experiments. The conditions are described in the text.

5) The enzymic inactivation of CoA with spleen extracts (100mg. acetone powder per ml.) and intestinal phosphatase. Tubes were set up as follows.

	1	2	3	4
1ml. spleen extract	+	+	−	−
1ml. boiled spleen extract	−	−	+	−
1ml. (about 100 units CoA	+	−	+	+
1ml. .9% NaCl	−	−	−	+
1ml. H20	−	+	−	−
4% MgCl2 (1 drop)	+	+	+	+

When intestinal phosphatase was used the pH was

brought to 8.5 with NaOH and instead of concluding the incubation after ninety minutes by boiling, it was continued for approximately fifteen hours at 37°C.

6) Citric acid synthesis.

The synthesis of citric acid was studied under conditions described in the text. Generally the final volume was 4.5ml. At the end of one hour's incubation at 37°C deproteinisation was carried out with 10% trichloracetic acid and filtration.

Estimations.

1) Bioassay of Acetylcholine.

After incubation the samples were treated with 1ml. of 0.3 N HCl, heated to boiling and then rapidly cooled. The samples were assayed within five days.

The acetylcholine content was assayed on the frog rectus muscle which had been previously treated with eserine (1in 100,000). All precautions outlined by Feldberg (1945), Feldberg and Hebb(1947) and Feldberg and Mann (1945-6)were taken to avoid distortion of the results by substances in the sample known to sensitise the muscle. Many trials were undertaken with the specific chemical method described by Hestrin(1949). This was found to be an excellent procedure for 0.5 -5mg of pure acetylcholine but quite unpredictable at the lower levels of acetylcholine. With tissue extracts the blank values were very high and I was not able to devise adequate controls for its use here.

2) Assay of Sulphanilamide.

The incubated mixtures were deproteinised with 4ml 5% trichloroacetic acid. 1ml. samples were treated according to the precedure of Bratton and Marshall(1939) - 1(Naphthyl) ethylene diamine was used to produce the colour. Acetyl sulphanilamide

was calculated by difference.

3) Assay of Citric Acid.

The procedures of Krebs and Eggleston(1943) and of Buffa and Peters (1949) were used.

4) Assay of Oxaloacetic acid.

The method of Friedmann and Haugen (1943) was used. Ethyl ether was used to extract the hydrazone. The absorption spectrum of various samples was determined by Dr. J. Fegler.

4) Assay of Phosphate.

The total, acid hydrolysable and free phosphate was separated according to the procedure of Eggleton and Eggleton (1929). The estimation of phosphate was carried out according to Fiske and Subbarov (1925) and where indicated in the text, according to Berenblum and Chain (1938).

Chromotographic Procedures.

For phosphorus containing compounds the method of Hanes and Isherwood (1949) was used. The solvent propanol-ammonia was used. Whatman No. 1 paper was washed with 2N HCl and 2N acetic acid and then several times with distilled water. "Versene" (ethylene diamine tetra acetate sodium salt) was added to the solvent in a concentration of 40mg.%.

The method of Cavallini et al (1949)

was used for chromatography of carboxylic acid dinitro phenyl hydrazones. Butanol-water was used as the developing solvent. The isolated dinitrophenyl hydrazone was dissolved in 0.01M phosphate buffer, pH 7.2 and approximately 25µg. in 0.1ml. applied as one spot to the paper.

RESULTS.

Part One.

Part 1.

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The Mechanisms of Ach Synthesis.

In Part 1 the mechanism of the synthesis of Ach by saline extracts of acetone dried brain and the effects of ATP, acetate, citrate and the coenzyme of acetylation will be discussed. The experimental section is arranged to demonstrate that the synthesis of acetylcholine is dependent, at least in part, on two separate systems, viz. an enzyme utilising acetate and one utilising citrate.

Acetylation of choline	Acetylation of sulphanilamide	Differences
Enzyme derived from acetone powder	Enzyme derived from acetone powder	—
Cysteine	Cysteine	—
Coenzyme of Acetylation	Coenzyme of Acetylation	—
K^+	K^+	—
A ATP	ATP	—
Citrate	Citrate	—
Mg^{++}		+
	Acetate	+
pH optimum 7.4 at 37	pH optimum 7.4 at 37	—

Table 1

The effect of acetate on brain acetylcholine synthesis in the presence of dialysed enzyme, ATP, cysteine and the coenzyme, under various conditions.

μ gAch formed in 1 hour at 37° per gm. acetone dried brain.

Additions	No. of Experiments	Average value	Range
ATP, 10 units CoA	4	500	320- 610
ATP, 10 units CoA and acetate	7	580	345- 650
ATP, 10 units CoA and citrate	6	1100	1000-1400
ATP, 10 units CoA and citrate and acetate	7	1050	1000-1400

TABLE 2

Part 1.

Experimental Results.

A. Inadvertent addition of acetate.

We have already noted in the introduction the system described by Kaplan and Lipmann (1948) for the enzymic acetylation of sulphanilamide is remarkably similar to the system described by Feldberg and Mann for the acetylation of choline. In Table 1 are listed the similarities. The third column shows the important difference that whereas the choline system apparently utilises citrate as a source of acetyl radicals the sulphanilamide system more logically utilises acetate. According to Kaplan and Lipmann citrate is added to their system only to bind Ca^{++} which activates the ATPase present in their enzyme extract.

In view of this apparent similarity, tests were performed with the choline acetylating system to determine whether it was even more comparable. Table 2 demonstrates that acetate when added alone or in combination with citrate exerts no significant effect on the amount of Ach synthesised. It is obvious however that even in the absence of citrate and added acetate there is still a very significant amount of synthesis.

Accordingly, it was suspected that acetate was being added inadvertently to the choline acetylating

TABLE 3

The effect of various additions on the acetylation of sulphanilamide by 1 ml. of the enzyme solution.

μg sulphanilamide acetylated.

Additions	Average value
Citrate, acetate, ATP, CoA	130
Citrate, ATP, CoA	130
Acetate, ATP, CoA	95
ATP, CoA	47
CoA	nil

TABLE 4

Conditions as in Table 3 except that the enzyme was dialysed against 0.02M $NaHCO_3$ with and without cysteine.

Additions	Dialysed enzyme	Dialysed against cysteine
Citrate, acetate, ATP, CoA	74	117
Citrate, ATP, CoA	78	124
Acetate, ATP, CoA	47	67
ATP, CoA	30	52
CoA	nil	nil

system. Because the sulphanilamide acetylating system is simpler and the estimations are more readily carried out we started by making a comparison of the effect of the addition of citrate, acetate and ATP. (Table 3)

It is readily seen that even in the absence of citrate and acetate a large amount of acetylation still takes place. We could not be certain of the chemical purity of the ATP or of the enzyme extract. Table (4) shows the effect of 48 hours dialysis of the enzyme extract. There is a decrease in the acetylating power of the enzyme but this could be largely reversed when the dialysis was conducted against cysteine. Probably this decrease in acetylation is due to oxidation of the SH groups of the enzyme.

It seemed probable therefore that the ATP, which is prepared with the nucleotide precipitant, barium acetate, contained contaminating acetate. Table (5) shows that this was in fact so, since when the nucleotide was prepared with barium nitrate, or when the acetate prepared ATP was reprecipitated with barium nitrate after washing with water, there there was no acetylation.

It soon emerged however, that not only could the acetylation of sulphanilamide take place when acetate was added with the purified ATP but, that some acetylation took place when citrate and acetate-

TABLE 5

Effect of acetate-free and acetate contaminated ATP on sulphanilamide acetylation.

μg sulphanilamide acetylated/ml. of enzyme/ 2 hours at 37°.

Additions	Result
CoA	nil
CoA, acetate-free ATP	nil
CoA, acetate-free ATP, acetate	52
CoA, acetate-contaminated ATP	43

TABLE 6.

The effect of magnesium ions on sulphanilamide acetylation in the presence of undialysed enzyme citrate, ATP and CoA.

μg sulphanilamide acetylated per ml. of enzyme per 2 hours at 37°.

Addition	Result
Acetate-free ATP, citrate	40
Acetate-free ATP, citrate, 0.001M mg	68
Acetate-free ATP, citrate, 0.002M mg	73
Acetate-free ATP, citrate, 0.003M mg	68

TABLE 7.

The disappearance of citrate during the course of incubation for sulphanilamide acetylation.

μg citric acid disappearing during 2 hours incubation at 37° per ml. of dialysed enzyme.

Boiled enzyme	Live enzyme
43	661
72	673

2 mg. of citric acid was added in a volume of 0.2ml. by a Conway microburette. Final volume, 2ml.

TABLE 8.

The effect of various conditions on the breakdown of citric acid by pigeon liver enzyme.

μg citric acid disappearing during 2 hours incubation at 37° per ml. of dialysed enzyme.

Additions	Breakdown
	Average of 3 experiments
Citrate 2 mg.	50
Citrate, .0.1M phosphate buffer	46
Citrate, 0.002M Mg^{++}	72
Citrate, 0.002M Mg^{++}, ATP, .05M	216
Citrate, ATP .05M	106
Citrate, Mg^{++}, ATP, CoA 10units	650
Citrate, Mg^{++}, CoA	140
Citrate, CoA	65
Citrate, Mg^{++}, ATP, CoA, 1.6 mg. cysteine	688
Citrate, Mg^{++}, ATP, CoA, 3.2 mg. cysteine	670
Citrate, Mg^{++}, ATP, CoA, 4.8 mg. cysteine	648

Final volume 3.0ml.

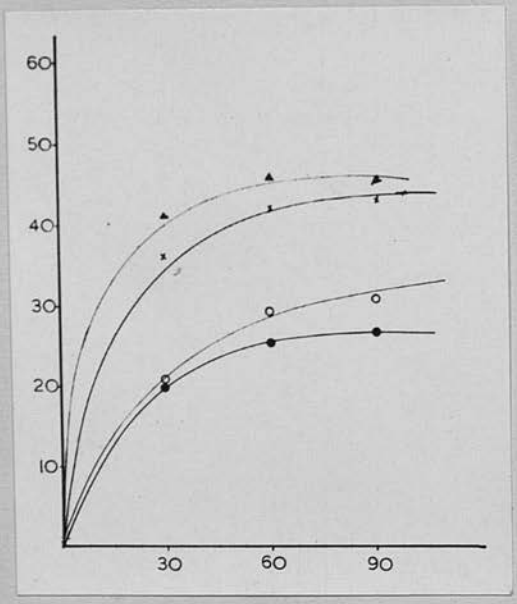


Figure 1.

The effect of citrate on the appearance of inorganic phosphate during the course of acetylation of sulphanilamide.

Ordinate:- μg inorganic phosphate appearing per 25mg. acetone powder.

Abscissa:- Time in minutes.

O no additions

X citrate 0·02 M.

▲ citrate and Mg 0·02M & 0·002M

● calcium 0·002M

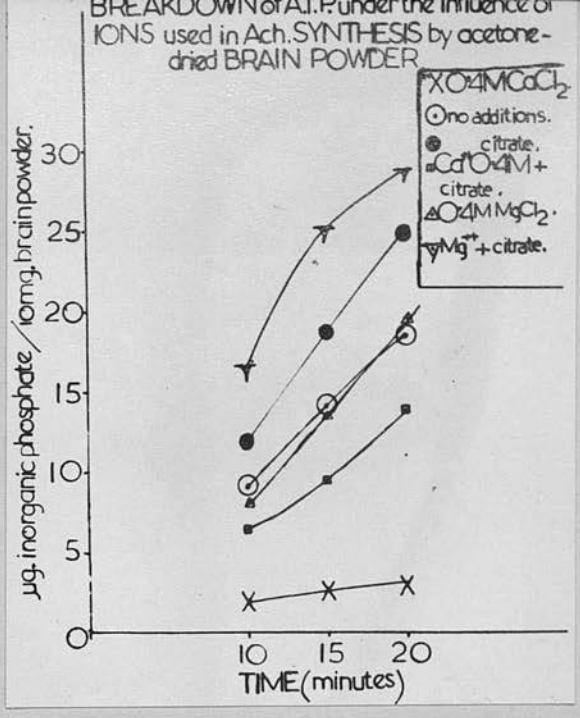


Figure 2.

The effect of citrate, calcium and magnesium on the production of inorganic phosphate during the course of acetylation of choline by brain enzymes.

Ordinate:- µg inorganic phosphate appearing per 10mg. acetone-dried brain.

Abscissa:- Time in minutes.

▽ 0.002M Mg^{++} and 0.02M citrate.

● 0.02M citrate

○ no additions

△ 0.002M Mg^{++}

X 0.002M Ca^{++}

□ 0.02 M citrate + 0.002 M Ca^{++}.

free ATP were added. In view of Feldberg's and Hebb's work showing that citrate was inactive in potentiating the synthesis of acetylcholine unless Mg^{++} was also added this was done and Table (6) indicates the potentiation obtained with magnesium.

Since Kaplan and Lipmann did not present experimental data in favour of their view that citrate was acting as an inhibitor of ATPase activity, the latter was measured. In contrast to their suggestion the addition of citrate enhances the appearance of inorganic phosphate, (Figure 1). Furthermore, inorganic phosphate production is even greater when magnesium is present and is actually diminished in the presence of calcium. These experiments could also be repeated with crude brain enzymes.(Figure 2)

Apparently citrate is metabolised by the crude enzyme extract. This was definitely established when citrate determinations were carried out at the end of incubation. The results were compared with a similar incubation mixture in which the enzyme had been boiled for two minutes. (Table 7) The breakdown of citrate was studied under the conditions listed in Table (8). The coenzyme, Mg^{++}, and ATP are necessary for the activity of this citrate dismuting enzyme. Section C describes more fully similar results withthe brain enzymes.

Fractionation of the pigeon liver extract was

TABLE 9.

Ammonium sulphate fractionation of the citrate dismuting enzyme in pigeon liver ectract.

μg citric disappearing per ml. of extract during 2 hours in the presence of Mg^{++}, ATP and CoA.

Enzyme fraction	Citric acid	Sulphanilamide acetylated
0-30% saturation	427	nil
30-80% saturation	0-30	64
Supernatant	nil	nil

carried out with ammonium sulphate at 0° C. The precipitate was collected by filtration after standing for approximately two hours in contact with $(NH_4)_2SO_4$. The enzyme utilising citrate is precipitated rather early. (Table 9). It could not be detected in the protein fraction precipitated above 30% ammonium sulphate saturation. The supernatant was brought to 80% and the resulting precipitate dissolved in 0.02 $NaHCO_3$ and then dialysed against 0.02M $NaHCO_3$ and 1 millimol cysteine. This preparation was tested for acetylating ability with sulphanilamide. In spite of about a 30% loss in activity this could be demonstrated. Further, citrate was without action here. (Table 9).

As little as 5µg of acetic acid could be detected by this simplified and purified system.

.....

Choline Acetylase.

The question whether acetate is utilised by the brain enzymes in the acetylation of choline was approached in a rather different way. It was to be expected from the results recorded in the above section that it would now be possible to demonstrate the accelerating action of acetate on the synthesis of acetylcholine. By analogy the following system should have demonstrated the action of acetate.

ATP purified	ATP purified
Enzyme	Enzyme
K^+	K^+
Coenzyme	Coenzyme
Cysteine	Cysteine
No acetate	Acetate
85µg/gm./hour.	210µg/gm./hour.

The results were however unconvincing because of the 1) small potentiation: 2) high base value.

Other reasons, recorded below, convinced Miss C.O. Hebb and me, however, that this system was analogous to the sulphanilamide system.

About this time Nachmansohn (1949) had reported the synthesis of a substance with acetylcholine-like properties by the brain extract. He based such a deduction on two facts.

1) That the bioassay of the incubated mixture yielded values approximately twice those obtained by a chemical method worked out by one of his pupils, Hestrin, (1949).
2) That this other bioactive material was synthesised in the absence of added choline by the protein enzyme fraction obtained between 25% and 36%

TABLE 10.

Experiments demonstrating our failure to synthesise a bioactive substance in the absence of choline-

Enzyme preparation	Ach µg/ml synthesised in 2 -3hrs. with choline	Ach µg/ml synthesised in 2 -3hrs. without choline	CoA Source
RP11. 16-30%	60	4	crude hog liver
RP13. 16-30%	44	2)	Hog liver sample
RP14. 25-36%	76	2.5)	from Dr. Lipmann
RP15. 16-25%	22	0.5-5)	Crude liver
RP15. 25-36%	53	1.5-5)	preparation
RP22. 25-36%	68	2	Purified from yeast (Balfour and Comline)

Note. The percentage ammonium sulphate corresponds 16-30% to 22-41% saturation, 16-25% to 22-36% saturation and 25-36% to 36-50% saturation.

TABLE 11.

Experiments to show that both acetate and citrate can act as acetyl donors for choline acetylation.

Acetylcholine μg per ml. synthesised in 3 hours.

Additions	Fraction 16%-28%	28%-36%	Notes
Acetate-free ATP	9	5	
Acetate-contaminated ATP	27.5	13	
Acetate-free ATP, acetate	125	27.5	
Acetate-free ATP, citrate	90	5	

These experiments were carried out on ammonium sulphate fractionated extracts according to Nachmansohn et al.(1949).

ammonium sulphate precipitation.

Because such an observation, if it were true, struck at the very foundations of our work, my supervisor, Miss Hebb, and I decided to try to repeat these observations. Table (10) summarises our results. We were quite unable to find evidence for the synthesis of this bioactive substance. Various samples, prepared by different methods, of the coenzyme were tried, and even the same preparation used by Nachmansohn was obtained by the courtesy of Dr. Fritz Lipmann. To summarise, in the absence of choline, neither ammonium sulphate fraction synthesised significant amounts of acetylcholine. In our hands, the chemical method was a failure because of its insensitivity, (it cannot detect less than 30 μg Ach), and it consistently gave values greater than those obtained by bioassay (the opposite to Nachmansohn's contention). The only explanation we can offer for the discrepancy between our results and Nachmansohn's is that he did not use the necessary controls in the bioassay of Ach. as advocated by Feldberg and Hebb.(1947)

However, Nachmansohn's system was of great interest since, for the first time, it clearly demonstrated the utilisation of acetate. There was no demonstrable synthesis when acetate was omitted from the incubation mixture (Table 11) provided that purified ATP was used.

It will be noticed that this system is purified in the sense that the 1st (0-16% ammonium sulphate fraction has been removed. By analogy with the fractionated sulphanilamide system where a similar fraction (citrate utilising) had been removed, it was to be expected that the next fraction would not synthesise Ach in the presence of citrate but in the absence of acetate.

This view was incorrect. It proved possible to divide the 16%-36% ammonium sulphate fraction into two parts (16%-25%, 25%-36%) the first utilising citrate preferentially, the second acetate. The second fraction (25% - 36%) was found to be inactive with added citrate and thus corresponded to the pigeon liver protein fraction soluble in 25% ammonium sulphate.*

This conclusive demonstration of the efficacy of acetate was the starting point for adumbration of the difference between the systems described by Nachmansohn on the one hand and that which we use on the other: see Table (12).

* corresponds to 35% ammonium sulphate saturation.

TABLE 12

A comparison between Feldberg and Mann's system and that of Nachmansohn et al. (1949).

enzyme	crude extract	fractionated extract
coenzyme	boiled brain extract	crude hog liver extract
cysteine	4.5mg.	18mg.
ATP Basalt	10. mg.	12mg.
KCl	6 mg.	9mg.
Eserine	.5mg.	0.1mg.
Acetate-Na	6 mg	4mg.
$MgCl_2$ $6H_2O$	4.0mg.	0.42mg.
$CaCl_2$	—	0.66mg.
Na_2HPO_4	—	1.2mg.
pH at 37	7.4	7.4
Total volume	4.5ml. with 0.9% NaCl.	3ml. with H_2O
Incubation time	1 hour	3 hours
choline	4mg.	12mg.

TABLE 13.

The effect of acetate on acetylcholine synthesis by dialysed saline extracts of acetone dried brain. Additions as under F and M (Table 12) except that acetate and ATP were omitted.

μg Ach synthesised per gm. per hour at 37°.

Additions	No. of experiments.	Average value.
Acetate-free ATP	4	85
Acetate-free ATP and acetate	4	210

Section B.

Further conditions to be observed in order that the utilisation of acetate by choline acetylase may be demonstrated.

Table (12) summarises the difference between Nachmansohn's acetate utilising system and the Feldberg and Mann system modified inasmuch as acetate was not added as contaminant of the ATP. It has already been noted that the latter system even in the absence of added acetate and added citrate is able to synthesise acetylcholine of the order of 100µg/gm.acetone powder/hour. These values cannot be accounted for by errors in the assay of acetylcholine. Furthermore when acetate is added to such a system, from calculations based on the Nachmansohn system, considerable yields of acetylcholine are to be expected. Table (13) demonstrates that we were able to show only a relatively small potentiation with the added acetate. In fact, probably the same amount as occurs in the Feldberg and Mann system when acetate is added as an ATP contaminant.

Theoretically one would expect no synthesis in the absence of added acetate and about 1000-1500µg/gm/hour in its presence.

The main difference between the two systems is

TABLE 14.

The effect of composition of enzyme extraction medium on the synthesis of Ach under the influence of acetate.

µg Ach synthesised per gm. per hour.

Additions	0.9% NaCl	*Nachmansohn'S	Nachmansohn's less cysteine
Acetate-free ATP	60	80	75
Acetate-free ATP and acetate	270	640	270

*Nachmansohn's extraction and dialysis medium comprises: KCl, 15gm.: $MgCl_2\ 6H_2O$, 200mg.: $Na_2HPO_4\ 12H_2O$, 2.1gm.: NaCl, 0.12gm. Cysteine, 120mg. per litre.

TABLE 15.

Extra cysteine added to the incubation medium has no effect on the level of acetylcholine synthesis with acetate as acetyl donor.

μg Ach synthesised per gm. per hour.

Additions	mg. Cysteine/4.5ml. incubation medium.					
	1	3	4.5	6	8	10
Acetate-free ATP,	40	30	60	60	72	55
Acetate-free ATP +acetate	280	320	270	280	260	300

*Enzyme extracted and dialysed against 0.9% NaCl.

in the nature of the medium used for extraction of the choline acetylase from the acetone-dried brain powder. Other differences responsible might be that:
1) Nachmansohn's system, in which the 0-16% ammonium sulphate fraction is discarded, may be free of an inhibitor (present in this fraction) of acetate utilisation: 2) differences in the ion composition of the incubation and extraction media.

Preliminary experiments were conducted in which a comparison was made between the amounts of acetylcholine synthesised when 0.9%NaCl was used to extract the enzyme and when the following medium was used. (KCl 15g; $MgCl_2 6H_2O$ 200mg; $Na_2HPO_4 12H_2O$ 2.1g; NaCl 0.12g; cysteine 120mg per litre) Table (14) summarises the results. Clearly a very significant difference exists.

The same experiment was conducted except that cysteine was omitted from the second extraction medium. Table (14) shows that there is an appreciable difference between the acetylcholine synthesised under the two conditions. Thus the synthesis of approximately 500µg Ach/gm/hr., under the action of acetate as acetyl donor is dependent upon the presence of cysteine in the extracting fluid. Experiments were conducted to determine whether increased concentrations of cysteine added to the incubation medium alone and not to the extraction medium were responsible. Table (15) shows that this was

TABLE 16.

Estimation of coenzyme of acetylation in dialysed undialysed and resin treated saline extracts of acetone dried brain.

Conditions.	Units CoA/mg. acetone dried brain.		
Undialysed	12	7.5	10
Dialysed 22hrs.	4.5	2	7
Dialysed 40hrs.	3.5	2.4	3
Resin treated 20min.	0.5	0.3	0.3
Resin treated 30min.	-	-	=

1 unit is defined as the amount of coenzyme needed to half reactivate the sulphanilamide acetylase system.

not so. Therefore, the demonstration of acetate utilisation by the brain enzymes is dependent on some protective or temporal function of cysteine in the extraction fluid.

It was now possible to show that approximately half the theoretically expected synthesis of acetylcholine with added acetate could be accounted for by the presence of cysteine in the enzyme extraction medium.

It had been noticed during the course of these experiments that the level of acetylcholine synthesis varied with the coenzyme preparation used. It was therefore decided to determine whether the acetate-utilising system was saturated with the coenzyme of acetylation.

If the system were not saturated with the coenzyme an increase in synthesis would be expected by raising the coenzyme concentration: similarly a decrease in the coenzyme concentration should decrease the amount of acetylcholine synthesised.

In order to construct the coenzyme activity curve a difficulty had to be overcome. It is, that the coenzyme of acetylation is precipitated by acetone as is the choline acetylase, and thus the coenzyme is included in the acetone-dried brain powder. The real difficulty arose when it was found (Table 16) that dialysis did not thoroughly remove the coenzyme. However during the course of purification

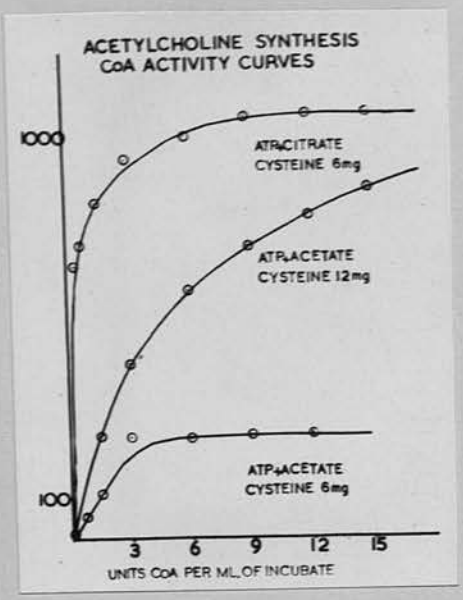


Figure 3.

The effect of the coenzyme on the synthesis of acetylcholine by resin-treated brain extracts.

Ordinate:- μg Ach per gm. per hour.

Abscissa:- units of CoA per ml. of incubate.

The upper curve is for citrate and ATP.

The middle curve is for acetate and ATP when the enzyme was extracted with saline containing 6mg. per ml.

The lower curve is for acetate and ATP. The enzyme was extracted with 0.9% saline only.

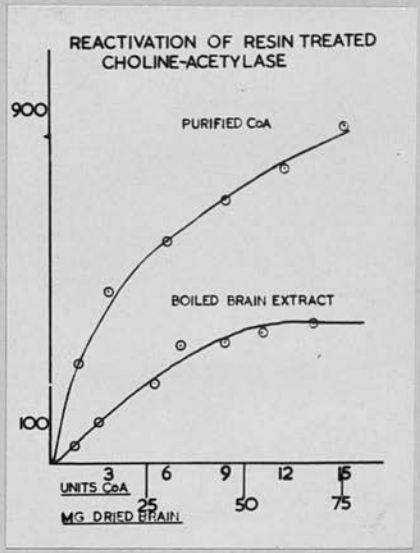


Figure 4.

Comparison of the effect of boiled brain extract and purified coenzyme on acetylcholine synthesis by resin-treated enzyme.

Ordinate:- µg of Ach per gm. per hour.

Abscissa:- units of CoA or mg. acetone-dried brain per ml. of incubate.

The Upper curve is for purified CoA.

The lower curve is for boiled brain extract.

Cysteine was added to the extraction medium.

of the coenzyme, described in Part 11, its anionic properties were apparent. A rapid and convenient method of removal of the coenzyme presented itself in the use of an anion-exchange resin. After such treatment the enzyme solution contained undetectable quantities of coenzyme. (Table 16)

When a partially purified coenzyme A preparation was added in various amounts to the resin-treated enzyme extract the construction of the Co A activity curve was possible. (Figure 3&4) The system became saturated at the coenzyme concentration of 25 units per ml. of incubation volume. It is thus evident that in the previous system coenzyme saturation had not been reached and this fact accounts for a lower synthesis of acetylcholine than would be expected on theoretical calculations. Indeed, an increase in the coenzyme concentration from 10 units per ml. to 30 units per ml. increased the acetylcholine synthesised to the expected level of 1050 μg/gm/hour.

Resin treatment of the enzyme extract was also important as a technique in resolution of the mechanism of acetylcholine synthesis for the following reason. It allowed the previously high base line values of acetylcholine synthesis (i.e. in the absence of added acetyl donors) to be reduced to zero. The fact that prolonged dialysis of the en-

TABLE 17.

A comparison of the effect of resin treatment and dialysis on the CoA content & acetylation of choline - the latter being studied in the absence of acetate and in the presence of acetate-free ATP.

μg Ach per gm. per hour.

	CoA content*	Ach value
***Dialysed enzyme	2.5	85
***Resin treated enzyme	nil	0**
***Resin treated enzyme, acetate	nil	790

*refers to enzyme CoA content

***30 units CoA was added to the incubation medium.

**the previously troublesome base line value has been reduced to zero.

enzyme extract did not reduce either the value for synthesis or the Co A level to zero suggested that an acetyl Co A complex existed which was only partially dissociable from the enzyme under the conditions of dialysis. Resin treatment however reduced both these values to zero as is shown when the treated enzyme was assayed on the fractionated sulphanilamide system for Co A content (Table 17).

Section C.

Citrate as an Acetyl Donor for Choline Acetylase.

The potentiating action of citrate on acetyl choline synthesis was observed by Feldberg and Mann (1945-6) and the dependence of this action on the presence of Mg^{++} ions was shown by Feldberg and Hebb (1947). There was no suggestion of the mechanism whereby citrate exerted this action. In 1946 Lipton and Barron (1946), on the basis of a further increase in Ach synthesis in the presence of semicarbazide and the known inhibition of synthesis in the presence of keto-acids, postulated the enzymic breakdown of citric to oxaloacetic and active acetate. This latter substance was postulated since these workers are in agreement with Feldberg and Mann in being unable to demonstrate a potentiating action on the synthesis of acetylcholine when acetate was added to the system.

It has now been possible to infer that citrate is acting as acetyl donor with some certainty since synthesis can now be demonstrated in a system which is acetate free. This was not possible before for the following reasons: the high values obtained in the absence of added citrate, for example, the value obtained with ATP and activator of Feldberg and Mann (1945-6), (later identified as CoA.) These values

TABLE 18.

The effect of various components of the incubation mixture on citrate breakdown by resin treated brain extracts.

μg of citrate breaking down per hour.

Additions*	Result
Citrate	-
Citrate, CoA (10 units)	-
Citrate, Mg(0.002M)	-
Citrate, CoA, Mg.	15
Citrate, ATP	-
Citrate, ATP, Mg	15
Citrate, ATP, Mg, CoA.	76
Citrate, ATP, Mg, CoA, semicarb.	92

Other components were added as in Table 1

*K^+ also found to be absolutely essential

	with K^+	without K^+
Citrate, ATP, Mg, CoA	76	17

TABLE 19.

The influence of citrate concentration on the breakdown of citrate by resin treated brain enzymes.

μg of citric acid breaking down per hour.

mg. citrate added*	Breakdown
1	0
3	0**
6	24
8	59
9	74
12	95

* incubation volume 4.5 ml.

** more than in the case of 1mg. but not determined.

indicated the presence of another acetyl source and citrate could well have been acting in this capacity, not as suggested by Lipmann and Kaplan (1948) as an ATP preserver.

This inference seemed more probable when it was found that the 16-25% ammonium sulphate protein fraction in contrast to the 25-36% fraction preferentially utilised citrate, as acetyl donor, in the system described by Machmansohn(1949).

Direct experimental evidence for this hypothesis is forthcoming under the following headings:-

1) The breakdown of citric acid.
2) The formation of an equivalent amount of keto acid.
3) The identification of the keto acid.

1) The Breakdown of Citric Acid.

Preliminary experiments carried out with various components of the incubation mixture, Table (18) showed that in order that citrate dismutation should take place the following components must be present:- enzyme, coenzyme, Mg^{++}, ATP and K^{+}. Further, it was shown (Table19) that a large amount of citrate must be present before any dismutation is evident. This fact indicated that we are here are dealing with an enzyme whose equilibrium is normally in the

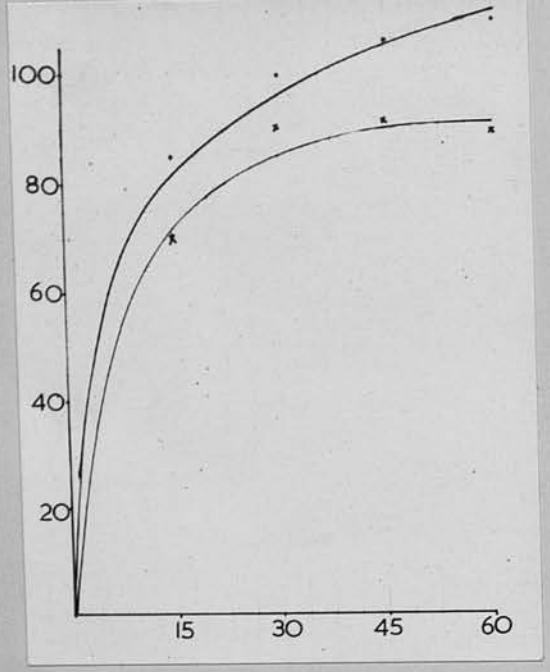


Figure 5.

The effect of semicarbazide on the breakdown of citrate by brain extracts.

Ordinate:- μg citrate disappearing per gm. of of acetone powder.

Abscissa:- Time in minutes.

The upper curve was obtained when 0.1M semicarbazide was added.

The lower curve was obtained when no semicarbazide was added.

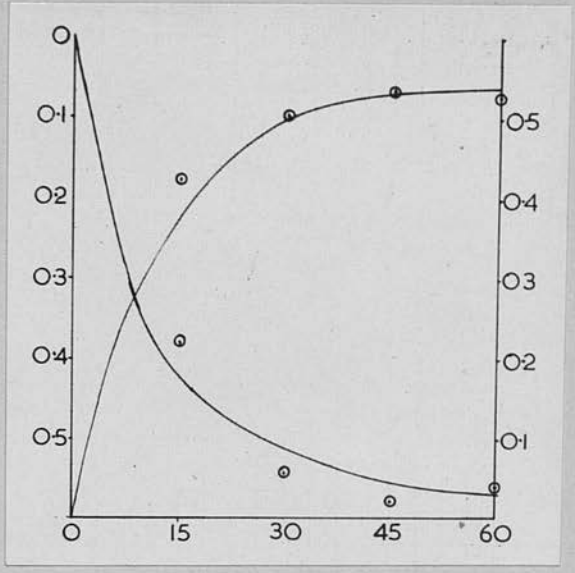


Figure 6.

The breakdown of citrate and the formation of an equivalent amount of oxaloacetate by brain extracts.

Left hand ordinate:- μM citrate disappearing.

Right hand ordinate:- μM oxaloacetate appearing per gm. per hour.

Abscissa:- Time in minutes.

	Light absorption of Phenyl hydrazones in alkali. Ratio. $\frac{420\ m\mu}{520\ m\mu}$
Pyruvic acid	1.28 - 1.40
α ketoglutarate	1.55 - 1.65
Oxaloacetic	1.28 - 1.40

Ethyl acetate as solvent.

After extraction of the phenylhydrazone solution with benzene which removes pyruvic acid hydrazone there was a 20% reduction in the light absorption of the hydrazone and the ratio 420 :520 $m\mu$ was then 1.40 indicating the presence of oxaloacetic acid hydrazone.

Table 29b.

opposite direction.

Figure (5) indicated the time course of the citrate dismutation and shows the increased dismutation when semicarbazide is also added. This increased dismutation in the presence of semicarbazide is probably accounted for by the fact that the semicarbazide by combination with the keto group of the dismutive product, prevents re-synthesis of citric acid.

2) The Formation of Equivalent Amounts of a Keto (Acid.

Figure (6) is a graph in which a comparison is made between the amount of citric acid dismuting and the appearance of a keto acid. When the amount of keto acid appearing is calculated on the assumption that it is oxaloacetic acid it will be seen that there is a close correspondence between the number of molecules of citric acid disappearing and the number of molecules of oxaloacetic acid appearing.

4) Identification of the Keto Acid.

Friedmann and Haugen (1943) recommend that in the identification of a particular keto acid a useful guide may be obtained when only one keto acid is suspected by comparing the ratio of absorption at 420 mμ and 520 mμ In Table is listed the ratio

for the keto acids and in the second column the ratio obtained in this experiment. An experiment was conducted as follows:-

200ml. of brain extract, 1000 units of CoA, 2.4gms. of sodium citrate, 300mgs. of ATP and Mg^{++} 0.002M were incubated at 37 C for 3 hours. Deproteinisation was effected with HCl and filtration. The filtrate was reduced to 50ml. in vacuo and 1gm. 2:4 dinitrophenyl hydrazine dissolved in 100ml. 2N HCl added. After standing at room temperature for 24 hours the crystalline precipitate was filtered off. The precipitate was dissolved in ethyl acetate and was recrystallised on the addition of benzene. The dinitrophenyl hydrazone was dissolved in 0.01M phosphate buffer (pH 7.2) and chromatographed on paper according to Cavallini, Frontali and Toschi (1949). There was a single yellow zone with an rF of 0.12 with butanol H_2O, thus agreeing very well with the value of 0.14 given by pure oxaloacetic dinitrophenyl hydrazone.

TABLE 20.

The acetylation of sulphanilamide by acetone fractionated pigeon liver extracts.

μg sulphanilamide acetylated per 2 hours per ml. of enzyme extract.

Enzyme fraction.	Acetylation value.	
0-40% fraction 0.2ml.	11	17
40-60% fraction 0.2ml.	nil	nil
60-100% fraction 0.2ml.	nil	nil
0-40%, fraction 0.2ml., + 40-60% fraction 0.2ml	69	62

TABLE 21.

The coupling of the crude brain extract with the sulphanilamide condensing enzyme from liver to acetylate sulphanilamide.

µg sulphanilamide acetylated per 2 hours per ml. of liver fraction.

Crude brain extract.	Liver fraction.	Acetylation.
-	0.2ml.	0
*0.2ml.(50mg)	-	0
*0.2ml.(50mg)	0.2ml.	43
*0.1ml.(25mg)	0.2ml.	62
**0.2ml.(50mg)	-	0
**0.2ml.(50mg)	0.2ml.	13
**0.2ml.(50mg)	0.2ml.	76

* acetate system, i.e. acetate, K^{+}, ATP, cysteine, CoA 8 units.

** citrate system, i.e. citrate, Mg^{++} K^{+}, ATP, cysteine, CoA 8 units.

Concentrations were adjusted to have the same value for the total volume of 1.5 ml. as for 4.5ml.

Note. The second citrate value was obtained by extracting the enzyme with 1ml..per 50mg., precipitating the enzyme in acetone and after air drying dissolving the acetone precipitate in 0.02M $NaHCO_3$.

Section D.

The coupling of brain enzymes and a liver enzyme to acetylate sulphanilamide.

The similarity between the choline and sulphanilamide acetylating systems having been established, it was decided to attempt a coupling between the two systems. The two enzyme systems resident in nervous tissue yield active acetate which acetylates added choline under the action of choline acetylase. There is not present in brain extracts an enzyme catalysing the acetylation of sulphanilamide. However, Lipmann et al. (1950), have described the preparation of a fraction from pigeon liver which contains the sulphanilamide condensing enzyme, i.e., the enzyme which catalyses the transfer of acetate from "active acetate" to sulphanilamide. The preparation of such a fraction (see experimental methods) has been confirmed and Table (20) shows that, for acetylation to occur in liver, another fraction, the 0-40%, has to be added in order to "prime" the added acetyl donors, citrate and acetate.

Table (21) demonstrates that the crude brain extract is able to replace the 0-40% acetone fraction obtained from pigeon liver. Also it is only in the presence of the 40-60% acetone liver fraction that sulphanilamide acetylation can occur.

It is evident that the same priming processes

occur in the crude brain extract as in the 0-40% acetone liver fraction. Further experiments may allow us to separate the choline condensing enzyme from brain and then reactivate this system with the priming systems derived from the 0-40% acetone fraction from pigeon liver.

These experiments lead us to the conclusion that at least two steps are involved in enzymic acetylation even when simple acetate is the acetyl donor. With citrate, it is possible that there are three steps: one to form acetate from citrate, one to activate the acetate and one to condense the activated acetate with the substrate.

.....

Section E.

The inactivity of acetyl phosphate and acetyl metaphosphate in choline acetylation.

An incubation mixture comprising the following components was set up: resin treated, cysteine extracted, enzyme: coenzyme: K^+: cysteine: choline: eserine. Acetyl phosphate and acetyl metaphosphate in various concentrations, in the absence and in the presence of acetate-free ATP were added and the resulting effect on acetyl choline synthesis determined.

Acetyl phosphate was originally suggested as a source of the acetyl group by Nachmansohn and Machado (1943) when reporting their discovery that ATP stimulated the acetylation of choline added to homogenised brain tissue. Lipmann (1946) similarly suggested acetyl phosphate as an intermediate in acetylations generally. This was not found to be the case. We were not surprised in view of both Comline(1948) and Lipton and Barron(1946) who found no actions on acetyl choline synthesis. Since these experiments were conducted it has now become rather generally accepted that acetyl phosphate is not a metabolite of animal tissues. There is evidence

for its utilisation by bacterial cells where an enzyme "transacetylase" seems to be important for its metabolism. The demonstration by Lipmann(1946) and Shapiro and Wertheimer (1945) that the enzyme acetyl phosphatase is extremely active in extracts from acetone-dried tissue and has an almost ubiquitous distribution, probably accounts for the extremely acid conditions encountered during incubation of this substance.

Acetyl metaphosphate.

This substance likewise was without action on acetylcholine synthesis. It had been hoped that this substance, being more dehydrogenated than acetyl phosphate, would contain a more labile phosphate bond which might be favourable for the synthesis of acetylcholine.

Section F.

The synthesis of citric acid by dialysed brain extracts.

The optimum requirement of the system synthesising acetylcholine with citrate as acetyl donor (Feldberg and Mann1945-6) is very high. For optimum synthesis approximately 12mg. of citrate have to be incubated with the saline extract from 50mg. acetone dried brain powder in a final volume of 4.5ml. Our demonstration that citrate exerts its action on acetylcholine synthesis by providing the acetyl radical and oxaloacetate as a side product suggests very strongly that the enzyme which dismutes citrate is the same one that synthesises citrate from oxaloacetate and acetate.

The equilibrium of the reaction

oxaloacetate + acetate $\rightleftharpoons$ citrate

under normal conditions is in favour of the condensation. If the citrate concentration is raised to a high level then some oxaloacetate and acetate formation is to be expected on theoretical grounds. Indeed, the section on citrate dismutation did show that the amount being broken down was not sufficient for optimal acetylcholine synthesis when less than 9mg. of citric acid was added. It is only when the 10mg. level is reached that the dismutation rate is

TABLE 22.

Citric acid synthesis by dialysed brain extracts and the necessity for the coenzyme of acetylation.

μM citric acid synthesised per hour at 37° per gm. acetone powder.

Additions.	Results
*Complete system.	6.7
No CoA.	0.63
5units CoA.	0.47
20units CoA.	1.4
50units CoA.	4.2
100units CoA.	6.7
No ATP.	0.6
No $Mg.^{++}$	1.0
No $K.^{+}$	1.3
No Cysteine.	6.4
Boiled enzyme.	0.43

* 5mg sodium acetate, 10mg oxaloacetic acid (neutralised), ATP 4.5mg, Mg^{++} 0.004M, K^{+} 6mg, 25mg NaHCO3, 50mg acetone powder, CoA, cysteine 4.5mg. Total volume 4.5ml.

high enough to keep the choline acetylation system saturated. Although, during the incubation period, with citrate levels of 1-10mg., sufficient acetate is forthcoming at a sufficiently rapid rate. Probably if one increased the length of time that the samples are incubated, then the optimum acetylation would result with much less citrate.

If these views are essentially correct (and Lipton and Barron (1946) present a similar hypothesis) then added support for them would be obtained by the demonstration of the presence of the citrate synthesising enzyme in the brain extract. Conclusive evidence would be obtained only by the separation of a single enzyme catalysing both reactions (condensation and dismutation).

In Table (22), there is listed the components found necessary to show citric acid synthesis in dialysed saline extracts of acetone dried brain. Oxaloacetate, acetate, the coenzyme of acetylation, ATP, Mg^{++} and K^{+} were all essential. The latter four components had been found essential for the breakdown of citrate. Cysteine, as in oxaloacetate formation exerted no significant action. The figures for citric acid synthesis probably do not represent the optimum values since no attempt was made to determine the optimum substrate concentration for the amount of enzyme present.

DISCUSSION.

The experiments of Part 1 show that the enzyme systems present in cell-free extracts of nervous tissue are able to synthesise acetylcholine by two, partially independent routes.

In order to demonstrate acetylcholine synthesis either of two systems may be used.

Enzyme	Enzyme
Coenzyme of acetylation	Coenzyme of acetylation
K^+	K^+
Acetate	Citrate
ATP (acetate free)	Atp (acetate free)
Eserine	Eserine
Cysteine high level.	Cysteine
	Mg^{++}

The second system is essentially that devised by Feldberg and Mann (1945-6)

The experiments we have reported demonstrate another, apparently more logical system that synthesises acetylcholine with acetate as acetyl donor. The failure of previous workers to show the efficacy of acetate is mainly due to the fact that they made additions of ATP which was contaminated with acetate. If such an addition had not been made then experiments conducted in the absence of citrate would have yielded zero or very low values. From this starting point some effect (up to 200µg Ach/gm/hr) with acetate would have been observed. No doubt

such a potentiation would have stimulated these previous workers to work out the optimum concentration for the coenzyme and for cysteine. Then, as it has been shown, the values for acetylcholine synthesis would be much the same as those reported for the citrate system.

It may be regarded as a fortuitous circumstance that the citrate system was capable of synthesising acetylcholine at all. Such a system presupposed either that the tissue extract could convert citrate to acetate or that acetate was known to be present in the ATP and that citrate had some other, indirectly potentiating action. It also becomes apparent that the choice of citrate was inspired inasmuch as the coenzyme requirement for optimum citrate activity was approximately one tenth of that required for acetate. This fact explains why citrate and ATP were effective in even a dialysed enzyme preparation. Sufficient amounts of the coenzyme remained after dialysis to activate the citrate system.

We have compared below the results obtained with both systems. Essentially they represent the difference between using acetate free ATP and acetate contaminated ATP and coenzyme free enzyme and the enzyme contaminated with coenzyme.

Source of Enzyme

Dialysed	Resin treated	Additions
20	0	ATP
-	0	ATP, acetate
470	0	ATP, CoA
-	*250-1050	ATP, acetate, CoA
40	0	citrate
400-900	0	citrate, ATP
310	0	citrate, CoA
1100	1040	citrate, CoA, ATP

Values expressed in μgAch per gm. per hour.

*This is not strictly true inasmuch as use of the acetate system requires the protection of some chamical groupings (presumably SH) by cysteine during extraction of the enzyme from the acetone powder.

Experiments have been conducted which show that this is indeed the case since an equal increase in the concentration of cysteine in the acetate incubation medium or in the citrate extraction and

and incubation medium has no effect on the synthesis of acetylcholine. We must therefore argue that the enzyme systems responsible for "priming" the added acetate, before it is"handed on" to choline acetylase, are very sensitive to oxidation.

It would be more difficult to argue that because the high coenzyme requirement of the acetate-utilising systems paralleled the increased cysteine requirement, a relationship existed here. If this were true, preincubation of the coenzyme with cysteine should enhance the synthesis, which it does not.

It is clear that a close relationship exists between the coenzyme and the cysteine requirement especially when the low coenzyme requirement for the citrate system is recalled together with this system's low cysteine requirement. One might suppose that the "priming" process referred to above is concerned with the activation of the coenzyme by an enzyme present in the brain extract which is sensitive to oxidation and thus requires the presence of a reducing medium. Further work is needed on this problem.

The inactivity of acetyl phosphate and acetyl metaphosphate is not surprising in view of Shapiro and Wertheimer's (1945) demonstration of the almost ubiquitous presence of acetyl phosphatase in tissue extracts and the doubtful position of acetyl phos-

phate in animal metabolism.

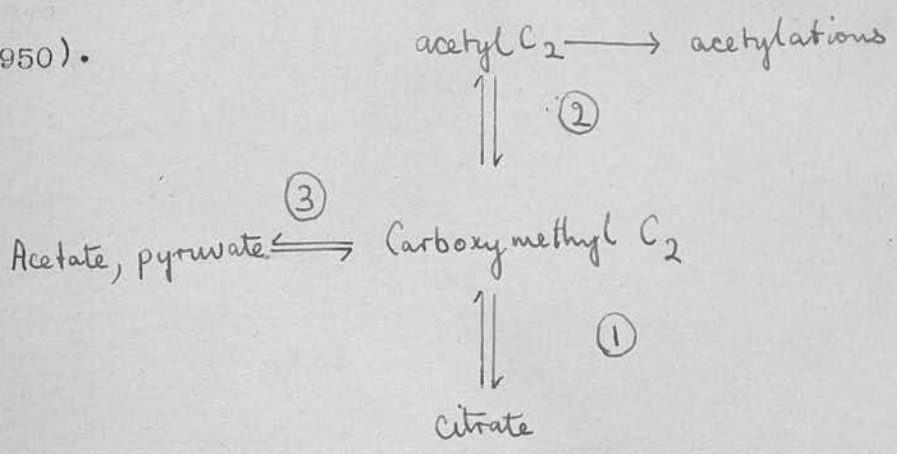
Our researches on the mechanism of citrate action in acetylcholine synthesis provides evidence for the suggestion made by Lipton and Barron (1946) that citrate acts as a source of acetyl radicals. It is possible to show the breakdown of citrate and the formation of oxaloacetate, and it is thought probable in view of the high citrate requirement and the presence of the citrate synthesis enzyme, that such a reaction is the reversal of normal equilibrium in favour of synthesis. Considering that it is this enzyme which controls the entry of acetate into the Krebs tricarboxylic acid cycle it is not surprising that it has a wide distribution. It is surprising, however, that the enzyme is present in cell free extracts; its presence is usually associated with the insoluble fraction of tissue. If the enzymes catalysing the conversions of the Krebs cycle are truly located in the mitochondria fraction, high speed centrifugation may be a way of removing this enzyme.

The recent work of Crandall and Gurin (1949) and Crandall et al. (1949) is highly relevant to the discussion of the principal problem arising out of this work, viz. the marked difference in coenzyme requirements of the citrate and acetate utilising systems. This marked difference suggests, since the amount of acetylation occurring is the same, that the

coenzyme utilisation occurs in the "priming" processes before the stage at which condensation with choline is reached.

The above investigators have studied the metabolism of C2 fragments derived from acetate, pyruvate and fatty acids by determining the distribution of radio-active carbon in acetoacetate produced by washed liver homogenates in the presence of labelled precursors with and without non-labelled precursors. It was concluded that there are two kinds of C2 fragment (acetyl and carboxymethyl). Acetate and pyruvate preferentially entered the carboxy methyl pool.

The following diagram is taken from V.R. Potter (1950).



Under similar conditions the enzyme catalysing the synthesis of citric acid was saturated at the CoA level of about 30 units per ml. of incubation mixture;-acetate was fully effective in the acetylation of choline at about the same level. However, in acetylation with citrate as the acetyl source

saturation was reached at the surprisingly low level of 3 units per ml. incubation mixture. In terms of the diagram, steps 1-2-4 requires 3 units and steps 3-2-4 and steps 3-1, 30 units. Such reasoning supports the postulate that step 3 requires about 27 units for saturation. However, so little is known of the chemistry of the coenzyme that it would be idle to speculate further. We would only mention that a similar argument can be applied in respect of cysteine; step 3 would appear to be readily inhibited by an oxidising atmosphere.

The general significance of the results recorded in Part 1 is difficult to assess. It could be reasoned that much of the work on the distribution of choline acetylase in the nervous system needs to be repeated since it is based on the presence of the citrate utilising system in the tissue extracts. It is probable however, because of the nervous system's high energy requirements, that the citrate synthesising enzyme is unlikely to be absent from any part. Indeed a comparison of the synthesising power of the two systems when the enzyme is derived from the cerebrum shows that the limiting factor is the concentration of choline acetylase. The problem could be resolved with certainty if it were known whether acetate or citrate is the normal acetyl precursor. From the energetic viewpoint acetate would appear to

fill this role since citrate first has to be synthesised and then broken down, processes which seem to require approximately twice the energy they would were acetate utilised directly. On the other hand, it may be argued that a lower coenzyme requirement would speak in favour of citrate. It would be safer to assume that alternative metabolic pathways exist and the route taken at any one time depends on the amounts of acetate or citrate in the metabolic pools.

Until it is shown that the concentration of the "citrate enzyme" is never a limiting factor in any particular tissue extract it would be unwise to deduce the presence or absence of choline acetylase. An extreme case may be cited:- the alternation of cholinergic and non-cholinergic neurons postulated by Feldberg (1951) could well be due to the presence and absence respectively of the citrate enzyme in the tissue extracts.

Part 1.

Summary

1) Both citrate and acetate can act as acetyl donors for choline acetylation by crude brain extracts.

2) In order to demonstrate the action of acetate three conditions have to be observed: a) that acetate is not added as a contaminant of the ATP: b) that the enzymes bringing acetate into reaction are protected during extraction from the acetone-dried powder by cysteine: c) that a ten times higher level of coenzyme than for the citrate system is used.

3) Citrate acts as an acetyl donor by breaking down to acetate and oxaloacetate. Mg^{++}, ATP and the coenzyme are essential for this reaction. The coenzyme requirement is low.

4) Citrate can act in a similar manner in the acetylation of sulphanilamide. Probably acetate and oxaloacetate are not the only products of citrate dismutation.

5) The citrate and acetate"priming reactions" carried out by the brain enzymes (in the absence of choline) can be coupled with a liver fraction (sulphanilamide condensing enzyme) to acetylate sulphanilamide.

6) At least two steps are involved in enzymic acetylation. Activation of acetate (probably a coenzyme acetate complex) and condensation with the acetyl acceptor are the main enzymic steps.

7) "Choline acetylase" includes more than one enzyme; it should only refer to the enzyme catalysing the condensation of active acetate with choline.

8) The validity of studies on the distribution of "choline acetylase" in the nervous system is discussed. No change in the ability of various parts of the system to synthesise acetylcholine is expected since the"citrate enzyme" probably has a universal distribution.

Part II.

The Purification and Structure of Coenzyme A.

Introduction.

R.J. Williams et al. (1936) first found that pantothenic acid participated in carbohydrate metabolism. About 1945, Feldberg and Mann (1945b), Nachmansohn and Machado (1943) and Lipmann (1945) showed that enzyme preparations catalysing on the one hand, the acetylation of choline, and on the other, the acetylation of toxic amides, contained a heat stable, partially dissociable component which was necessary for full activity. In Part 1 it is shown that the acetylation of choline is absolutely dependent on the presence of a similar substance. Evidence for the identity of this substance with Lipmann's coenzyme of acetylation is presented in Part 11.

Initial attempts to demonstrate the presence of a B vitamin in this factor were unsuccessful, because pantothenic acid, the vitamin component, is bound in such a way that it is not released by the usual methods used for preparing samples for microbiological assay. Acid hydrolysis of the active fraction did however yield appreciable amounts of β alanine. The coenzymatic activity of preparations of various degrees of purity paralleled the β alanine content (Lipmann (1947). More recently, it was shown that pantothenic acid can be liberated by digestion with two enzymes. Lipmann (1947) used intestinal phosphatase

and a liver enzyme and Comline (1948) takadiastase and an enzyme present in spleen extracts.

It was soon realised, however, that neither of the rather specialised acetylations studied could account for the general importance of the vitamin. Lipmann (1947) has shown that essentially all the pantothenic acid of animal tissues occurs in bound form, almost certainly the coenzyme of acetylation (CoA).

The metabolic importance of pantothenic acid is indicated by the following tabulation.

1) Reactions requiring CoA.

A. Formation of acid phosphates.

Acetic acid + ATP → phosphoryl acetyl intermediate + ADP.

B. Formation of esters.

Choline + acetic acid + ATP ⟶ ACh + ADP + H_3PO_4.

C. Formation of amides.

Sulphanilamide + acetic acid + ATP ⟶ acetylated amine + ADP + H_3PO_4.

D. Formate pyruvate interchange.

E. Hippuric acid synthesis.

F. Condensation reactions.

2acetic acid + ATP → acetoacetic acid + ADP + H_3PO_4.

Oxaloacetic acid + acetic acid + ATP ⟶ citric acid + ADP + H_3PO_4.

2) Conversions requiring pantothenic acid which can be demonstrated in vivo by the use of pantothenic acid antagonists, deficiencies and isotopes.

Acetic acid → fatty acids (bacteria).

Acetic acid → sterols (bacteria).

Acetic acid → aromatic amino acids (bacteria).

Acetic acid → $CO_2 + H_2O$ (yeast).

Glucose or pyruvate or lactate → $CO_2 + H_2O$ (liver).

Proteins or carbohydrates → fats (rats).

The structure of CoA is not known. Lipmann et al.(1950) have described the preparation of a fraction which on the basis of its pantothenic acid content and the rate at which it diffuses through sintered glass membranes is 30% pure. This preparation was obtained from 500 lb. liver with a yield of 625 mg. The components identified in this preparation were: pantothenic acid 11%: phosphorus 9%: adenine 18%: pentose 22%: some cystine and some glutamic acid. Subsequently, the preparation of a fraction from 10 lb yeast which, on the basis of its acetylating ability, was at least 70% pure will be described here.

Studies on the enzymatic degradation of the compound by Lipmann (1947) and Comline (1948) indicate that there are at least two linkages which must be cleaved before pantothenic acid is microbiologically available. Either enzyme treatment alone

renders the coenzyme inactive (each producing a diff-ent product). Neither enzyme by itself liberates pantothenic acid.

Baddiley and Thain (1951) have synthesised panto-lactone phosphate, pantothenic acid 2 phosphate and pantothenic acid 4 phosphate. None of these subst-ances can stimulate the growth of Acetobacter subox-ydans. The liver enzyme degradation product of CoA is here active. Baddiley concludes that either this active fragment is more complex, containing fur-ther substituents on the pantothenic acid residue, or that phosphate is linked to pantothenic acid through some other component.

Two other reports of pantothenic acid derivat-ives have appeared in the literature. King, Fels and Cheldelin (1949) have reported the concentration of a conjugate from liver and heart muscle which is not permeable to cellophane membranes. Williams, Hoff-Jorgensen and Snell (1949) have reported the concentration from bacteria of a factor containing pantothenic acid, yet not CoA, which is necessary for the growth of Lactobacillus bulgaricus.

In this work I have been fortunate in having at my disposal large amounts of a readily prepared frac-tion from yeast which is very active in coenzyme A activity. Dr. R.S.Comline of the Physiology Labora-

tory, Cambridge, was able to show (1948) the presence of acetylating activity in the phenol extracts of y yeast currently used as a source of flavine adenine dinucleotide. The procedure developed by him, more particularly the removal of FAD with collidine is described in the Experimental Section.

Use of this method and the use of spleen extracts (1948) to inactivate the coenzyme, have allowed a considerable purification of the coenzyme to be achieved and some pointers to its structure have been obtained. This work was undertaken as a necessary requisite before the physiological properties of the coenzyme in the nervous system could be determined.

Section A.

The identity of Feldberg and Mann's "activator" with the coenzyme of acetylation.

Comline (1948) presented good circumstantial evidence that the substance he had isolated from baker's yeast was identical with the coenzyme of acetylation prepared by Lipmann (unpublished method). Lipmann's preparation was active in both sulphanilamide and choline acetylation and of course contained pantothenic acid. Comline's preparation was only tested for its choline acetylating ability. It contained approximately 40µg pantothenic acid per ml. of concentrate. The pantothenic acid was only available microbiologically after a double enzyme treatment (spleen extract and takadiastase) and thus similar to Lipmann's CoA from which pantothenic acid is only released after a double enzyme treatment (avian liver enzyme and intestinal phosphatase).

In this work it has been possible to confirm the results of Comline and add the following observations:

1) That the yeast concentrate is active in sulphanilamide acetylation.
2) To establish the CoA activity on a dry weight basis.
3) To show that a brain extract (Feldberg and Mann's activator) may be concentrated in a different manner from yeast; it is active in both choline and

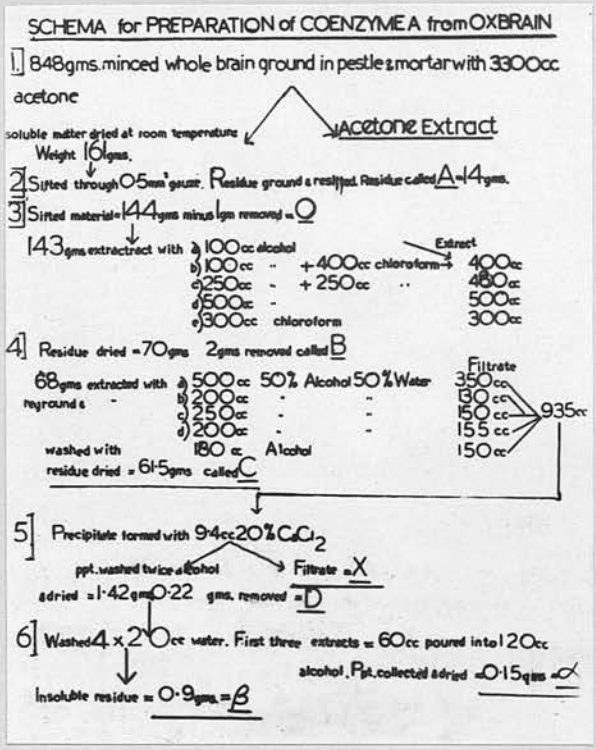

SCHEMA for PREPARATION of COENZYME A from OXBRAIN
1] 848gms. minced whole brain ground in pestle & mortar with 3300cc acetone
soluble matter dried at room temperature
Weight 161gms.
Acetone Extract
2] Sifted through 0·5mm² gauze. Residue ground & resifted. Residue called A=14gms.
3] Sifted material=144gms minus 1gm removed = O
143gms extract
a) 100cc alcohol
b) 100cc " + 400cc chloroform
c) 250cc " + 250cc "
d) 500cc "
e) 300cc chloroform
Extract
400cc
480cc
500cc
300cc
4] Residue dried = 70gms 2gms removed called B
68gms extracted with
reground &
a) 500cc 50% Alcohol 50% Water
b) 200cc " "
c) 250cc " "
d) 200cc " "
180cc Alcohol
washed with
residue dried = 61·5gms called C
Filtrate
350cc
130cc
150cc
155cc
150cc
935cc
5] Precipitate formed with 9·4cc 20% CaCl2
ppt. washed twice alcohol
Filtrate = X
dried = 1·42gms 0·22 gms. removed = D
6] Washed 4 x 20cc water. First three extracts = 60cc poured into 120cc alcohol. Ppt. collected & dried = 0·15gms = α
Insoluble residue = 0·9gms. = β

Figure 7.

TABLE 23.

Activity in Units per mg. of various preparations of the Coenzyme from Yeast.

CoA Units per mg.

	Apoenzyme Preparation.		
	3	4	7b
Coenzyme preparation 1	33	40	31
Coenzyme preparation 2	58	55	67
Coenzyme preparation 5*	22	19	20

* prepared from ox brain by the same method as for yeast.

TABLE 24.

Comparison of the activity of a coenzyme preparation when assayed with the crude liver and fractionated liver extract.

Units per mg.

	Crude extract	Fractionated extract
Coenzyme prep. 2	58	51

sulphanilamide acetylation. (Figure 7).

4) To apply Comline's method of yeast extraction to brain and obtain a fraction whose action on sulphanilamide acetylation cannot be distinguished from the yeast concentrate.

5) That both the brain and yeast concentrates are inactive after incubation with spleen extracts, for sulphanilamide acetylation.

Table (23) summarises the results of three experiments carried out with different apoenzyme preparations from pigeon liver. In each case the experiment was conducted in such a manner that an assay of CoA activity was made. These assay values are reported on a dry weight basis. (The coenzyme was dried by pouring the sample into twenty volumes of ice-cold acetone, washing with acetone and ether and drying in a desiccator). In view of the report of Lipmann et al. (1950) that some resynthesis of CoA from its split products occurs in the crude apoenzyme preparation and that resynthesis does not occur in the 40-70% saturated ammonium sulphate fraction, experiments were conducted on this point. Table (24) demonstrates that there is no evidence for the view that the yeast concentrate contains the degradation products of CoA.

Table (25) provide the experimental data on which

TABLE 25.

The coenzymic activity of an ox brain preparation concentrated according to Figure 7.

μg acetylated per ml. of enzyme.

	In sulphanilamide acetylation	In choline acetylation
No additions	0	8
1mg. concentrate	40	19
3mg. concentrate	110	36

* Feldberg and Mann system.

on which our conclusions have been based.

Convincing evidence for the identity of CoA with the activator is thus provided. Also we are able to decide the degree of purity attained by Comline's procedure. The best preparation assayed at 60 units/mg. Lipmann's best preparation (1950) has 130 units of sulphanilamide acetylating activity per mg. He judges this preparation to be 30% pure. Our preparations is therefore approximately 15% pure but the yield is about fifteen times greater.

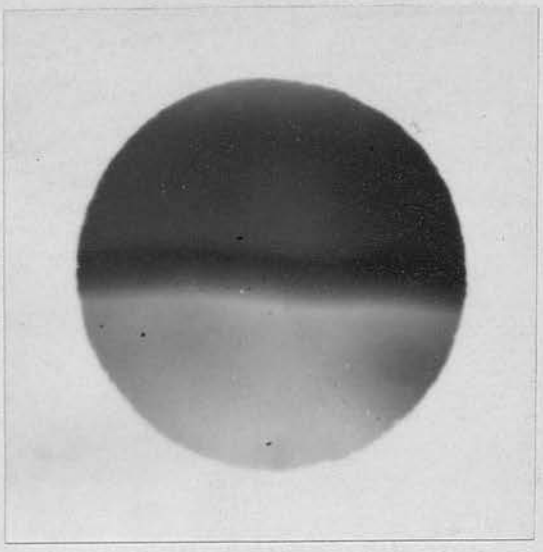


Figure 8.

Photograph taken under ultra-violet light of the last stage of collidine extraction of the yeast concentrate. Under white light both layers appear homogeneous.

The upper layer fluoresces green-yellow.

The lower layer fluoresces bright blue.

Section B.

The Blue Fluorescence of Coenzyme Concentrates.

Comline (1948) examined the concentrated yeast extracts (Experimental Methods) on a partition chromatogram on paper with collidine as a developing solvent. He showed that the accelerating action of yeast extracts on choline acetylation was not associated with flavin adenine dinucleotide but with a band giving a blue fluorescence under ultra-violet light. However, this blue fluorescing band was not a good criterion of chemical purity since the band did not move appreciably from the starting point with collidine as solvent. Another observation, the inactivation of the coenzyme by digestion with spleen extracts, and the movement of the blue fluorescing zone after such treatment suggested that the coenzyme of acetylation was responsible for the blue fluorescence under ultra-violet light. Whether this is correct or not, these experiments were useful in forming the basis for further purification of the coenzyme. Direct extraction of the yeast extracts with collidine removes the flavine derivatives. The collidine is readily removed from the coenzyme A concentrate by shaking with twenty volumes of ether.

Figure (8) is a photograph taken under ultra-

TABLE 26.

The chromatography of the coenzyme with phenol-KCl.

	Units
Blue fluorescing zone 0.09	1.5
Zone rF 0.11	3.9
Zone rF 0.2	0

violet light of the last extraction of the coenzyme A concentrate with collidine. Under white light both layers are homogeneous but under ultra-violet light the upper (collidine) layer fluoresces green and the lower (water) layer, bright blue.

In order to decide whether the blue fluorescence is associated with the coenzyme it was decided to develop paper chromatograms with a solvent in which the coenzyme was known to be soluble. Phenol was the obvious choice since it is used as the first step in the concentration of the coenzyme from yeast. Indeed it did move the blue fluorescing zone but the zone was too diffuse to cut out and elute to test for acetylating ability. A better solvent system was found when the phenol was equilibrated with 0.05% KCl at pH 4.5 (HCl). Such a solvent system moved the zone discretely with an rF value of 0.09. This zone was eluted or in some cases simply incubated with sulphanilamide acetylation system to determine the coenzyme A activity (Table 26). Although it contained some coenzyme, most (70%) of the activity was located immediately in front of the blue zone (rF 0.11). This evidence was not considered good enough to make possible a decision on the association of CoA activity with the blue fluorescence.

However, at a much later stage in the work, when the coenzyme had been purified by some of the steps

to be reported in a later section, it was decided to attempt the synthesis of "active acetate" on the assumption that the latter substance is the acetylated form of coenzyme A. The gas ketene, generated by the pyrrolysis of acetone, was selected as the acetylating agent because it has been demonstrated that little racemisation occurs if the solution is maintained faintly alkaline. Further, as will be shown later, one of the reactive groups of the coenzyme only makes itself evident under alkaline conditions.

The treatment with ketene was remarkable in changing the blue fluorescing solution into one which fluoresced green under ultra-violet light. On acidification the blue fluorescence returned in a much more marked form. Whereas very little blue fluorescence was noted when the original solution was diluted ten times, after the ketene the solution could be diluted five hundred times when the blue fluorescence was still strong. The solution was no more active in acetylation than it had before this treatment.

Paper chromatography of the ketene treated solution with collidine as developing solvent revealed one blue fluorescing band with an rF of 0.10 which corresponds to the band moving in collidine after spleen enzyme digestion of the coenzyme preparation, first described by Comline (1948).

Some control experiments were performed since it was suspected that some acetone vapour came over with the ketene gas. It was found that all the above effects could be duplicated by treatment of the coenzyme sample with acetone under alkaline conditions. No change in intensity of the blue fluorescence was observed after addition of acetone under acid conditions. In view of various reports in the literature (Holf and Perlzweig 1943, 1944) it was considered that an N substituted nicotinamide (probably N-methyl nicotinamide) was responsible for the original blue fluorescence and that treatment with acetone under alkaline conditions yielded the highly fluorescent condensation product 1 methyl-3 carboxylamide-6 pyridone which fluoresces green under alkaline and blue under acid conditions. It is further suggested that the spleen extracts contain a quinine oxidising enzyme which catalyses the same reaction. The ultra-violet absorption spectra of these compounds is being carried out. It was found (Table 27) that n butyl alcohol removed both fluorescent compounds from the coenzyme solution without any loss of activity .

Confirmation of the non-identity of the substance responsible for the blue fluorescence and the coenzyme of acetylation was forthcoming when a paper chromatogram with propanol/ammonia was developed. The blue

TABLE 27.

The effect of butyl alcohol extraction on yeast concentrates.

	Units CoA
Untreated sample	14/ml.
Butanol extracted sample	12/ml.
*Butanol layer	0/ml.

*Butanol was removed under reduced pressure and the residue taken up in water and washed with ether.

fluorescing zone had an rF value of 0.42 whereas the coenzyme (recognised as a phosphorus-containing compound and by its acetylating activity) had an rF value of 0.55. (Table 28).

TABLE 28.

Chromatography of the coenzyme with propanol-ammonia. Azone with an rF value of 0.52-0.55 was cut out and incubated with the CoA assay system.

Zone	µg sulphanilamide acetylated / ml. enzyme/2 hours.
0.71 (Control)	0
0.52-0.55	107

Section C.

The Presence of a Thiol Grouping in the Coenzyme.

Three suggestive lines of evidence led to the concept that the coenzyme of acetylation contains a reactive SH group. In the first place it was demonstrated in Part 1 that the acetate utilising system for choline acetylation requires more coenzyme for optimum activity than the citrate utilising system. This increased coenzyme requirement may be associated with the protective function of cysteine required during the enzyme extraction. The citrate utilising system has an extremely low coenzyme requirement. It is doubtful if cysteine is required at all here.

Secondly, when the breakdown of citric acid by brain enzymes was being studied, an attempt was made to obtain evidence for the intermediate formation of phospho-enol-oxaloacetic acid. It was thought, in view of this system's requirement of Mg^{++} K^{+} and ATP that it was analgous to the reversible system, pyruvate, + ATP $\rightleftharpoons$ phospho-enol pyruvate, + ADP.

If this were so, providing that phospho-enol-oxaloacetic was not much more unstable than phospho-pyruvate, we should have got positive results with the alkali-iodine treatment devised by Lohmann and Meyerof (1935) to estimate phospho-pyruvate. Indeed

TABLE 29.

An attempt to demonstrate the formation of phosphoenoloxaloacetic acid.

Additions	ml.N/300 I_2
1) Complete system (10 units CoA)	2.8
2) Complete system (20 units CoA)	5.1
3) No citrate	2.8
4) No ATP	2.8
5) No Mg.	2.9
6) No CoA	1.1
7) Enzyme boiled before incubation	2.8

TABLE 30.

A comparison of the reducing values and acetylating activity of two preparations of the coenzyme.

Preparation	units/ml	ml0.0033N I_2
7	116	27.1
8	74	18.9

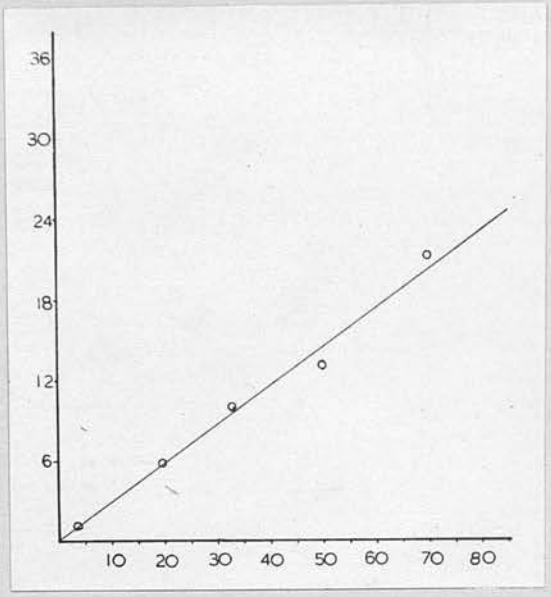


Figure 9.

Graph showing the direct relationship between the acetylating activity of the coenzyme and its reducing value to iodine.

Ordinate:- ml. 0.0033N I_2.

Abscissa:- units of CoA.

we did get considerable iodine titrations but it was not possible to demonstrate the formation of equivalent amounts of phosphate.

Table 29 indicates the conditions under which the titrations were carried out. It soon became apparent that almost all of the iodine reducing value was represented by the coenzyme content of the incubation mixtures. The coenzyme has no reducing value if it is not exposed to alkaline conditions before titration. When the conditions were standardised (CoA sample made normal with respect to NaOH and 1cc0.1N I_2 added, and held at room temperature for 30 minutes). Then (1cc 2N HCl was added and the residual I_2 titrated with0.003N $Na_2S_2O_3$) it was possible to use this procedure to estimate the coenzyme. Figure 9 is a graph in which CoA activity (acetylation of sulphanilamide) is plotted against the reducing value to iodine. In Table 30 a comparison is made between the acetylation and reducing values in terms of CoA activity from various samples of collidine-extracted yeast concentrates. This method became the basis for the preparation of 70% pure coenzyme.

The behaviour of the coenzyme with alkali and iodine suggested that a sulphydryl group was involved. The nitroprusside test was faintly positive; the colour faded very rapidly, but after

treatment with cyanide the coenzyme yielded a positive reaction.

The specific spot test for sulphur described by Fritz Feigl (1949) was applied. Sulphur catalyses the extremely slow reaction between iodine and azide, releasing nitrogen. This test gave a positive result. According to Feigl if a positive result is given by the iodine azide test then a decision can be made between the groupings $>C=S$, $>C-SH$ by the following procedure. The coenzyme solution is warmed with sodium acetate and iodine and then tested with the iodine-azide reagent. If the result is still positive then the grouping C=S is present, and if negative the grouping C-SH is present (owing to the oxidation of the sulphydril group to a disulphide linkage which does not react). After such treatment the result was only faintly positive.

Section D.

The purification of the Coenzyme of Acetylation.

The partial purification of coenzyme A from yeast has been reported by Comline (1948). Since this method has not yet been published it is reported in full in the experimental section. This method does not yield consistent results. The assay values for several preparations range from 21 to 60 units of coenzyme activity per milligram. The ratio of the activity to the pantothenic acid content (kindly carried out by Dr. E. Kodicek) corresponds fairly well with the value of 0.7µg per unit reported by Lipmann et al. (1950). More consistent results have been obtained here probably as a result of allowing the three phenol extractions of the Koschaft to take place over a much longer period. Each extraction with phenol took five to seven days. This procedure increased the coenzyme content to between 80 and 100 units per mg.; the total yield for 10lbs of baker's yeast was approximately 300mg. For reasons already stated (Section B) 1cc. of the extract containing 5.4mg. of solids was extracted twice with three volumes of n butyl alcohol. After removal of the butyl alcohol the water layer was dried and yielded 2.4 mg. The coenzyme activity was determined as 168 units per mg. Thus the butyl

alcohol extraction increased the activity from 82 to 168 units per mg. without appreciable loss.

About this time Lipmann (1950) published a method of preparing coenzyme A assaying at 130 units per mg. and 30% pure. It was thought that the application of the methods used by him (barium and acetone fractionation) to our sample might substantially increase the purity. This was not so, and we had to conclude that the phenol, silver precipitation, collidine, butyl alcohol treatment removes much the same impurities as Lipmann's barium and acetone fractionations. Since Lipmann starts with 500lbs. of liver and finishes with 625mg., our procedure, yielding 300mg. from 10lbs. yeast, is very much more efficient.

The only established chemical characteristic of the coenzyme is its acidic nature. It is precipitated by heavy metals, probably through the SH group, and an increase in hydrogen ion concentration increases its solubility in non-aqueous solvents. The observation in Part 1 that it is strongly adsorbed by the anion exchange resin De Acidite E confirmed this and offered another method for further purification.

Cohn et al. (1950) have shown that anion exchange resins can be used in much the same way as starch columns in chromatography to separate certain adenylic

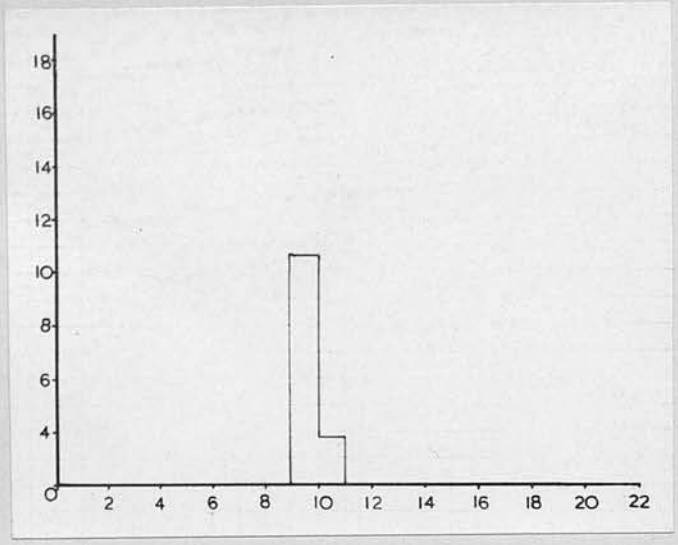


Figure 10.

Graph showing the reducing value of various 10ml. fractions collected from a column of the anion exchange resin De Acidite E.

Ordinate:- ml. 0.001N I_2 required per 10ml. fraction.

Abscissa:- fraction number (each has a volume of 10ml.).

Seventy four fractions were collected in this experiment but the reducing material (equivalent to ml 0.0033N I_2) did not appear.

acid derivatives. Instead of using an organic solvent the column is developed with an acid which displaces the one which is in chemical combination with the resin. If the yeast concentrate is applied to the column in a very small volume, it can be developed, as in a chromatogram, as a discretely moving zone.

The simplest prodedure we could devise was as follows:- the anion exchange resin De Acidite E was soaked in 5% NaOH overnight and then backwashed with 0.15N HCl for 24 hours. Then a mixture of 0.15N HCl HCl and 0.1N NaCl was allowed to pass through the column at a flow rate of 1ml. per minute for five hours. 50mg. of the crude coenzyme (8400 units) dissolved in 1ml. of the developing solution was carefully applied to the column and the effluent was collected in 10ml. fractions (column length 27cms. by 1.5cms. diameter). 1ml. of each fraction was held for 30 minutes in N NaOH, brought to neutrality and titrated with 0.005 N I2 from a Conway horizontal micro-burette. The graph constructed from these results is shown in Figure 10. Fractions 9 and 10 were combined and poured into ice cold acetone at pH 3.5 and the mixture kept at 0° C overnight. The resulting white precipitate was dried with ether and after drying was found to weigh 17.1mg. This material assayed at 310 units per mg. According to

Lipmann's calculations this corresponds to a purity of 70%.

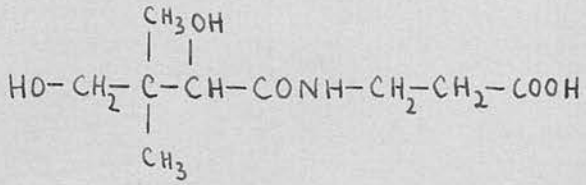
Paper partition chromatography with propanol-ammonia revealed one phosphorus containing zone with an rF value of 0.55. A zone in a similar position on another untreated strip of paper was cut out and incubated with the sulphanilamide system when acetylation resulted.

Baddiley and Thain (personal communication) have shown that Lipmann's preparation is not homogeneous with respect to phosphorus - two zones can be shown by paper chromatography.

Section E.

The Structure of Coenzyme A.

The results to be reported in this section are mainly based on reports in the literature of the components identified in coenzyme A concentrates. Lipmann (1950) concludes from studies on the enzymatic release of pantothenic acid from coenzyme A concentrates that two chemical bonds have to be broken.



There are three points of attachment in pantothenic acid, the carboxyl and two hydroxyl groups. Among the other constituents identified are adenine, ribose and phosphate in molar ratios of 1 : 1 : 3. Baddiley (personal communication) has been able to demonstrate the presence of pantothenic acid 4 phosphate in both acid and alkali hydrolysates by paper chromatography. He could find no trace of either the 2 phosphate or the diphosphate. In these hydrolysates he could also show the presence of adenosine 5 phosphate. Since Lipmann has shown that the coenzyme is inactivated by a highly specific and purified potatoe pyrophosphatase

it seems probable that part of the coenzyme can be formulated thus:

Adenine-ribose-phosphate-phosphate-pantothenate.

Recently Snell et al. (1950) have shown that another pantothenic acid conjugate, the Lactobacillus bulgaricus factor is a molecule comprising two fragments, pantothenic acid and β mercapto-ethanolamine. They have been able to synthesise the factor with the carboxyl group of pantothenic acid in amide linkage with β mercapto-ethenolamine. This substance has the same rF value in pyridine / water as the isolated fraction.

We have synthesised a conjugate of pantothenic acid and β mercapto-ethanolamine by the following route:

A. β mercapto-athanolamine according to Wenker (1935).

β amino ethyl sulphuric acid was prepared by thermic dehydration of mono-ethanolamine acid sulphate. 175gms. of β amino ethyl sulphuric acid was distilled with 546gms. 40% NaOH and 160ccs. of distillate collected. On addition of potassium hydroxide 40cc . of base separated. This was dried repeatedly with potassium hydroxide, then with sodium and finally fractionated. The yield of ethylene imine, boiling at 54-55° was 14gms. 10gms. of ethylene imine in 90ccs. of absolute alcohol was added dropwise (3-4 hours) into 50ccs. of chilled absol-

ute alcohol surrounded by an ice bath, while a current of H_2S was passed through the well stirred mixture. The solution was concentrated under reduced pressure to 20ccs. in vacuo. A white crystalline solid separated which was washed with petroleum ether and air dried. Yield 9gms. It melted at 95.7°C.

1.01gms. of calcium pantothenate was dissolved in 15ccs. H_2O and treated with 0.255gms. of oxalic acid. The precipitate was removed by centrifugation and the supernatant removed under vacuum. The resulting dark brown oil was dried at 60°C over H_2SO_4 for 2 days. 352mgs of pantothenic acid and 180mg β mercapto ethanolamine were heated together in a sealed tube under vacuum at 100°C for 90 minutes. The seal was broken and the tube heated under vacuum at 70°C to remove unchanged to mercapto-ethanolamine for 90 minutes. The resulting brown crystals were dissolved in water and extracted with n butyl alcohol- The butyl alcohol was removed under vacuum and the resulting semi-crystalline mass dried over $CaCl_2$.

Titration with iodine showed it to be 81% pure and 2% free amino nitrogen was present.

Paper partition chromatograms developed with Butanol/H_2O revealed one sulphur containing zone which did not correspond with β mercapto-ethanolamine. The zone was recognised by spraying the dried paper with iodine-azide reagent (Chargaff 1948)

A brief note by Snell et al. (1950) on the synthesis of the Lactobacillus bulgaricus factor has appeared. The essence of the method is that the amide linkage between the carboxyl group of pantothenic acid and the amino group of β mercapto ethanolamine is brought about by ester condensation.

Since we had no definite proof that the previous method did in fact yield the LBF factor we have used a method based on Snell's report.

1.1952gms. of calcium pantothenate was dissolved in 10ml. of water and 0.316gms. of oxalic acid (hydrated) added. The calcium oxalate so formed was removed by centrifugation and the water removed from the supernatant under reduced pressure. The brown oil was dried over $CaCl_2$ for five hours.

Diazomethane was prepared in ether from nitrosomethylurea (methylamine hydrochloride and urea and nitrous acid).

The dried pantothenic acid was dissolved in 10ml. methyl alcohol and treated with an excess of diazomethane in ether. The mixture was allowed to stand over anhydrous potassium sulphate for three hours at room temperature and then filtered. The filtrate was evaporated in vacuo to a constant weight of .8048gms.

.8048gms. methyl pantothenate were dissolved in 20ml. methyl alcohol and refluxed with .277gms.

β mercaptoethanolamine and 0.001 gms. acetamide. At the end of four hours the mixture was cooled and held for one hour at room temperature in the presence of KOH in methanol to hydrolyse any remaining ester. After neutralisation (HCl in methanol) the solvent was removed and the residue partitioned between n butanol and water. The butanol was removed and the residue dried to constant weight.

$C_{11}H_{22}O_4N_2S$	Calculated	N	10.07%	SH	11.9%
	Found	N	11.0%	SH	12.0%

Paper partition chromatograms developed with butanol-water revealed a sulphur containing zone (rF 0.37) which corresponded with a zone obtained from an acid hydrolysate of CoA (15minutes at 100° C in N HCl). Sulphur was recognised by spraying the air-dried papers with the iodine azide reagent of Chargaff (1948). It is thus concluded that the LBF factor forms a component part of the coenzyme molecule.

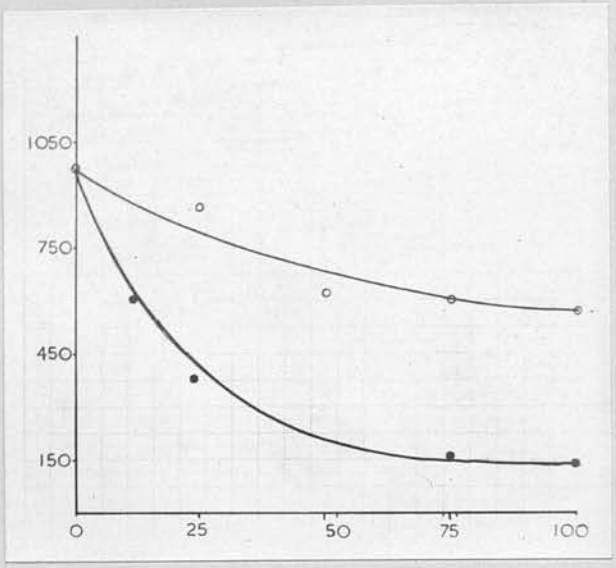


Figure 11.

The effects of spleen extracts on brain acetylcholine* synthesis in the presence of two levels of Coenzyme A.

Ordinate:- µg Ach synthesised per gm. per hour.

Abscissa:- mg. acetone-dried rabbit spleen added as a saline extract.

Upper curve:- 50 units of CoA per ml. incubate added.

Lower curve:- 30 units per ml. incubate added.

*Acetate system used.

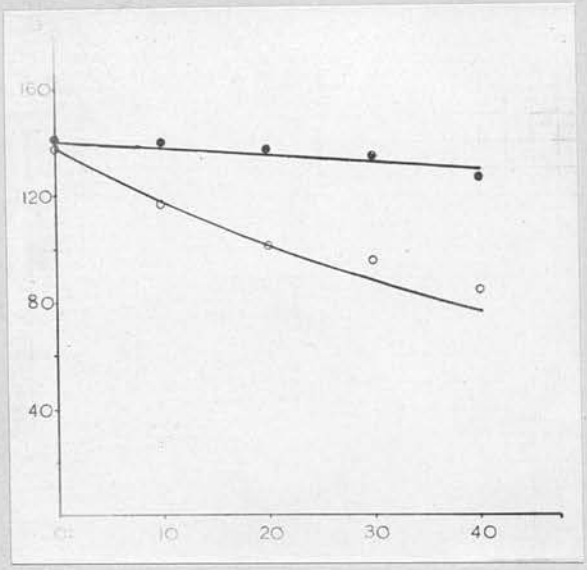


Figure 12.

The effect of spleen extracts on liver sulphanilamide acetylation in the presence of two levels of Coenzyme A.

Ordinate:- μg sulphanilamide acetylated per ml. enzyme per 2 hours.

Abscissa:- mg. acetone-dried rabbit spleen added as a saline extract.

Upper curve:- 12 units CoA per ml. incubate added.

Lower curve:- 4 units CoA per ml. incubate added.

Section F.

Enzymic Breakdown and Resynthesis of the Coenzyme.

Comline (1948) showed that one of the enzymes present in a saline extract of acetone dried spleen was extremely active in reducing acetylcholine synthesis by brain extracts. Since he could also show that a similar extract was a necessary part of the system needed to release pantothenic from the coenzyme, he concluded that the spleen enzyme reduces acetylcholine synthesis by destroying the coenzyme. It seemed very probable, therefore that the spleen enzyme would have the same action on the more pure concentrates. This point was tested, a comparison being made between the inclusions of spleen extracts in the brain system acetylating choline and in the liver system acetylating sulphanilamide. (Figures 11 and 12) Very different results were obtained. In the brain system the spleen enzyme reduced acetylcholine synthesis at two levels. With the liver system only a slight reduction in the amount of sulphanilamide acetylation was observed at low coenzyme levels and no significant effect was recorded at high coenzyme levels. This observation was confirmed by incubating the spleen enzyme and coenzyme alone and then observations were made on both systems.

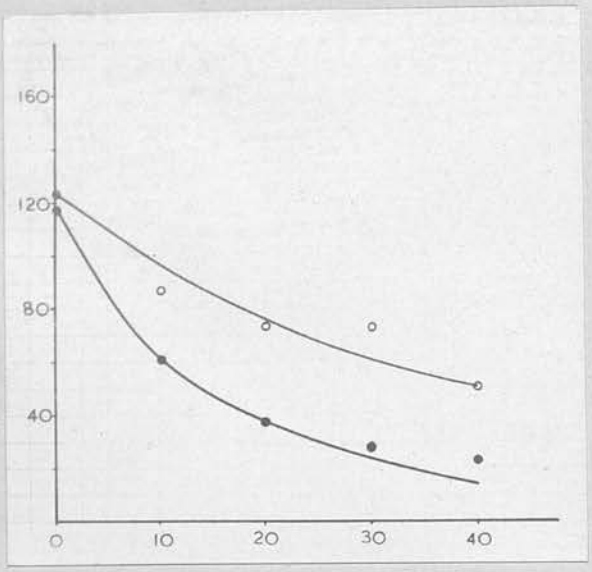


Figure 13.

The effect of spleen extracts on liver sulphanilamide acetylation (35%-80% ammonium sulphate fraction) at two levels of Coenzyme A.

Ordinate:- μg sulphanilamide acetylated per ml. of enzyme per 2 hours.

Abscissa:- mg acetone-dried rabbit spleen added as a saline extract.

Upper curve:- 12 units of CoA per ml. incubate added.

Lower curve:- 4 units of CoA per ml. incubate added.

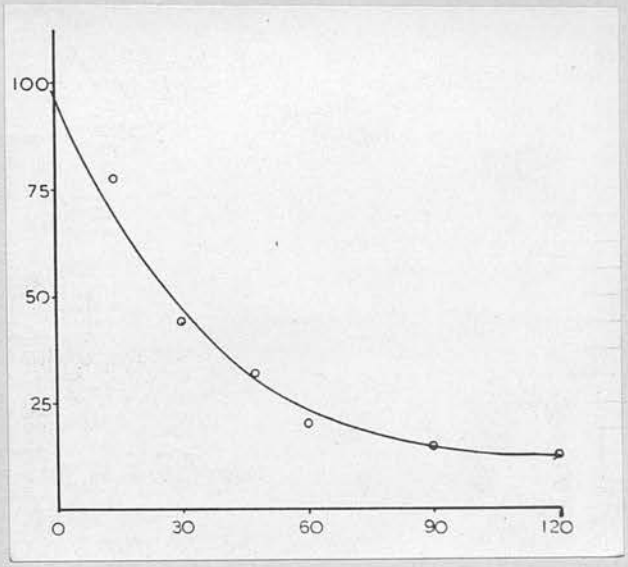


Figure 14.

The effect of preincubation of 100 units of CoA with rabbit spleen (100mg.) on the CoA remaining as assayed by the fractionated liver enzyme.

Ordinate:- % CoA remaining.

Abscissa:- Time in minutes.

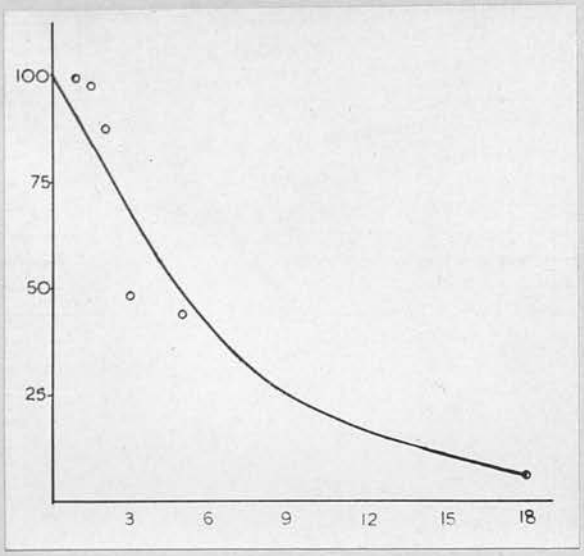


Figure 15.

The effect of digestion of 220 units of CoA by a crude intestinal phosphatase. The remaining CoA was assayed with the crude liver enzyme for sulphanilamide acetylation.

Ordinate:- % CoA remaining.

Abscissa:- Time in hours.

The spleen incubation inactivated the coenzyme for acetylcholine synthesis but not for the sulphanilamide system. We conclude that with the liver system, enzymic resynthesis of the split products of spleen digestion was occurring. Control experiments were conducted to make sure that the spleen extract did not contribute anything that could be resynthesised into the coenzyme. These were negative.

When a similar experiment was conducted with the ammonium sulphate fractionated liver enzyme entirely comparable results with the brain system were obtained; i.e. the spleen extract markedly reduced the amount of acetylation taking place. The coenzyme was then incubated with the spleen enzyme alone. After boiling, these extracts were assayed for remaining coenzyme with fractionated liver system (Figures 13,14). It is concluded that the spleen enzyme splits the coenzyme into products which can resynthesised by the 0-35% saturated ammonium sulphate fraction of the liver extract.

Intestinal phosphatase has been used by Lipmann (1947) as part of the procedure for the release of pantothenic acid from the coenzyme. We have used a crude preparation of this enzyme to determine whether the products of coenzyme digestion can be resynthesised by the liver enzyme. Figure 15 shows that digestion of CoA with intestinal phosphatase inactivates the coenzyme and since the assay was conducted

TABLE 31.

The inhibitory effect of the LBF factor on re-synthesis of the coenzyme by the liver enzyme (unfractionated).

	μg sulphanilamide acetylated/ml. of enzyme/hr.
Spleen digested CoA.	120
Spleen digested CoA, 50μg LBF	11
Spleen digested CoA, 75μg LBF	0

with the unfractionated liver enzyme the products of digestion were different from those of spleen digestion.

These experiments are interesting since they provide a basis for further work on the structure of the coenzyme.

Since the lactobacillus bulgaricus factor (pantothenyl-amino-ethane-thiol) has been shown to be a component part of the coenzyme molecule, this substance was added to the unfractionated liver enzyme and incubated with ATP, acetate, K^+ and sulphanilamide. No acetylation was observed. The same result was obtained when it was incubated with the degradation products from intestinal phosphatase digested CoA. When it was incubated with the spleen digested CoA there was a marked inhibition of the resynthesis. (Table31). From these experiments we can only conclude that the LBF factor is not one of the spleen degradation products but it might be one of the intestinal phosphatase degradation products.

Paper partition chromatograms have been prepared for phosphorus containing compounds according to the method of Hanes and Isherwood (1949). With propanol-ammonia we have been able to obtain quite good differentiation of various phosphorus containing zones. The purest preparation of the coenzyme moves a discrete band with an rF value of 0.55.

We have compared this zone with the phosphorus containing zones obtained after spleen and intestinal phosphatase digestions of the coenzyme. Neither enzyme preparation contained phosphorus nor did the boiled enzymes release phosphorus compounds when incubated with the coenzyme.

The most striking difference between the distribution of the phosphorus containing zones of the two incubations was the presence of inorganic orthophosphate in the intestinal phosphatase digest. Both were alike however in releasing the same adenosine phosphate compound. In these early chromatograms both digests had zones with the same rF value as the untreated coenzyme. When the development time was increased it became apparent that this was not true for the CoA, spot moved more rapidly than the spleen spot which moved more rapidly than the intestine spot. It thus became possible to identify the number of spots produced on digestion. The spleen treatment gave two spots, one an adenylic acid derivative and another phosphorus spot (rF 0.52). The intestine treatment yielded three spots - one the same adenylic acid derivative as the spleen, inorganic orthophosphate and a spot with an rF value of 0.50.

Discussion.

It has been possible to show that the procedure developed by Comline (1948) is a very effective method od of concentrating the coenzyme of acetylation. Use of this method, but with a more prolonged phenol extraction and the further procedures, butyl alcohol and chromatography, have allowed a considerable concentration of the coenzyme to be effected. The best preparations are approximately 70% pure (based on the assay value in units per mg. and Lipmann's estimate of the molecular weight). Lipmann's best preparations was obtained from 500lbs. of liver in a yield of 625mg. and it assayed at about 30% purity. Although yeast contains only one third of thenamount of coenzyme present in liver, the yield is 25 times greater on a weight basis, i.e. 10lbs. of yeast yields as much coenzyme as 250lbs. of liver. It is almost certain that this difference results from the long delay which must ensue from the removal aof liver and the heat inactivation of the coenzyme autolysing enzymes. Yeast cells are normal organisms right up until the time that they are dropped into boiling water.

Only very small quantities of the purest coenzyme have been obtained so far but it seems likely that more could be obtained if a larger resin column were used. On theoretical grounds there are no reasons for supposing that the coenzyme could not be com-

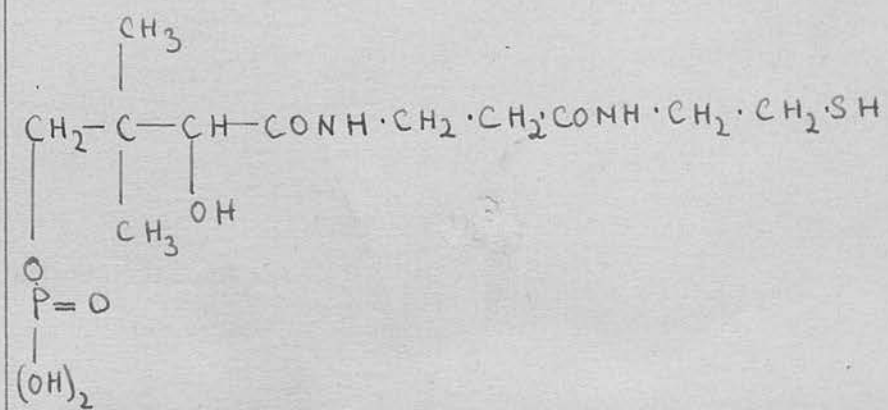
pletely isolated by this method. When this is attempted it will be necessary to collect small fractions of the eluant. Since this step will increase the number of samples to be analysed for reducing value by titration with iodine, it may be better if the latter technique were abandoned in favour of paper chromatography. Titration with iodine is arduous and unsatisfactory in the sense that the coenzyme is not the only iodine reducing material present in the yeast concentrates applied to the column. Probably the most convenient method would be to use the new, rapid assay method of Handschumacher et al. (1951).

The work described on the paper chromatography of the coenzyme, and the degradation products produced by the spleen enzyme and intestinal phosphatase are very interesting since they provide a basis for the elucidation of the structure of the coenzyme. Both enzyme digests of the coenzyme release a phosphorus containing compound which corresponds with one of the zones obtained when a preparation of ATP is developed with propanol-ammonia. It is not easy to identify which adenine compound is released since on development of ATP with this solvent hydrolysis takes place and discrete zones, corresponding to adenosine triphosphate, adenosine diphosphate and adenosine monophosphate, are not obtained. It will

be possible to resolve this difficulty when pure adenosine derivatives are available since presumably only a small amount of hydrolysis will occur on development and the pertinent zone will be recognised by its greater size.

Intestinal phosphatase releases inorganic phosphate from the coenzyme whereas the spleen enzyme does not. The other phosphorus containing zone released by the intestinal phosphatase has an rF of 0.50 which is different ftom the zone (rF 0.52) obtained on spleen digestions. (N.B. These values do not appear very different but if development is allowed to proceed for a long time each zone can be readily separated from the other.)

Since the products of spleen digestion can be resynthesised into the coenzyme by an enzyme present in pigeon liver extracts and since N(pantothenyl) amino-ethane-thiol has been shown to be a component part of the molecule which cannot be resynthesised under the same conditions it is suggested that the above compound with phosphate substituted on the fourth carbon of the pantothenic acid residue



is the phosphorus containing fragment produced by spleen digestion.

This hypothesis will be more tenable when it can be shown that this zone can be resynthesised to the coenzyme.

If this postulate is correct then the synthesis of the coenzyme may be regarded as analogous to the enzymic mechanism of synthesis of flavine adenine dinucleotide. Schrecher and Kornberg (1950) have shown that flavine adenine dinucleotide is synthesised by enzymic catalysis of the reaction.

Riboflavin phosphate,+ATP ⇌ FAD,+inorganic pyrophosphate.

In their experiments ATP could not be replaced by ADP or AA and with riboflavin and ATP very little occurred.

By extension of the analogy, the coenzyme of acetylation may have the basic formula:

Adenine-ribose-phosphate-①-phosphate-②-pantothenate-ethanolamine SH

Intestinal phosphatase breaking bonds 1 and 2.

Spleen enzyme breaks bond 1.

However this formulation does not agree with the fact that intestinal phosphatase liberates a phosphorus containing compound (rF 0.5) which is not adenylic acid or inorganic phosphate. A further argument against this formulation is that Lipmann (1950) has

If this solution were then active in acetylating sulphanilamide or choline when the condensing enzyme alone was added, convincing evidence for the presence of the intermediate coenzyme-acetate complex would be obtained.

Without exception, in the other known coenzymes, containing a member of the B vitamin group, the active, functional, grouping is a property of the vitamin part of the molecule. This fact suggests that the hydroxyl of pantothenate is the carrier of the acetyl radical. With the acetyl group located in this position it would be necessary to postulate acetyl or hydroxyl group activation by ATP prior to actual union. Perhaps also in favour of this hypothesis is the fact that the terminal SH group of the coenzyme would be able to anchor the coenzyme to the enzyme which is also sensitive to oxidation.

If we consider the thiol grouping of the coenzyme as the acetyl carrier portion of the molecule, it is perhaps easier to see the role of ATP as an energy donor for enzymic acetylation. Supposing that the following reaction can occur:

$$-CH_2-S-H + CH_3COOH \rightarrow -CH_2-S-OCCH_3 + H_2O$$

followed by:

$$-CH_2-S-OCCH_3 + ATP \rightarrow -CH_2-\underset{\displaystyle PO_3H_2}{\underset{|}{S}}-OCCH_3 + ADP$$

the sulphur-acetyl bond becoming labile, and thus an

demonstrated three molecules of phosphorus per molecule of pantothenate. Since his preparation is not chromatographically homogeneous with respect to phosphorus, this argument is not very cogent.

If the molecule does contain a third phosphate radical (and our evidence suggests this is so) it could only be attached to the γ hydroxyl of pantothenate or to the thiol grouping of the molecule. Baddiley and Thain (presonal communication) have tested the former possibility by looking for pantothenic acid 2 phosphate in acid hydrolysates of the coenzyme. They in fact found only pantothenic 4 phosphate, and since these phosphate derivatives are quite stable, conclude that the coenzyme does not contain pantothenate 2 phosphate.

When we consider the possible position for a third phosphate radical it would be advisable to bear in mind the acetyl transferring function of the coenzyme. The experiments described in Part 1, particularly those concerned with the coupling of the brain and liver enzymes to acetylate sulphanilamide, suggest very strongly that a coenzyme-acetate complex is formed which is an active acetylating agent in the absence of ATP. A more direct experiment to test this point would be to incubate the brain enzyme, coenzyme, acetate and ATP, then boil the incubate and destroy the remaining ATP with an ATPase preparation.

effective acetylating agent. Some evidence for this postulate has been obtained by treatment of the coenzyme with alkali. Under such conditions the iodine value of coenzyme appears (probably the thiol group is formed) and some phosphate is released which is detected by the extraction method of Berenblum and Chain (1938) and is thus not molybdate catalysed.

It is hoped that further work on these problems will elucidate the structure of the coenzyme.

Summary.

1) Evidence is presented to prove the identity of the activator with the coenzyme of acetylation.

2) The coenzyme is not responsible for the blue fluorescence described by Comline (1948).

3) A method of purification, based on the acidic and reducing properties of the coenzyme is described.

4) Acid hydrolysates of the coenzyme contain a sulphur fragment which is probably identical with the Lactobacillus bulgaricus factor.

5) Digestion of the coenzyme with an enzyme derived from rabbit spleen releases two phosphorus-containing fragments which can be resynthesised to the coenzyme by a liver enzyme.

6) Digestion of the coenzyme with a crude intestinal phosphatase releases three phosphorus-containing fragments which cannot be resynthesised to the coenzyme under the conditions described.

7) The structure of the coenzyme is discussed.

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